Role of phosphoinositide 3-kinases in mast cell activation

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ABBREVIATIONS

4E-BP1	eukaryotic initiation factor binding protein
5-HT	5-hydroxytryptamine
Abl	abelson protein tyrosine kinase
AD	atopic dermatitis
AdB	adaptor-binding site
AID	activation-induced cytidine deaminase
APC	allophycocyanin
APCs	antigen-presenting cells
AR	allergic rhinitis
ATM	ataxia telangiectasia mutant
ATP	adenosine triphosphate
Bad	Bcl2-antagonist of cell death
BCAP	B cell PI3K adaptor protein
Bcl-Xl/Bcl-2	Bcl-Xl/Bcl-2-antagonist causing cell death
BCR	B cell receptor
BH	breakpoint-cluster region homology
BM	bone marrow
BMMCs	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
Cbl	casitas B-lineage lymphoma
CCR	CC chemokine-binding receptor
CFSE	carboxyfluorescein succinimidyl ester
CR3	complement receptor 3
CSR	class-switch recombination
CTG	CellTracker Green

CTMC	connective tissue mast cells
CXCR	CXC chemokine-binding receptor
Cyc D1	cyclin D1
DAG	diacylglycerol
DC	dendritic cell
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNP	dinitrophenyl human serum albumin
DP	prostaglandin receptor
dsRNA	double-stranded ribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EEA1	early-endosomal autoantigen 1
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
eIF2B	eukaryotic initiation factor 2B
ELISA	enzyme linked immunosorbent assay
EtOH	ethanol
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
Fas L	Fas ligand
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
FN	fibronectin
FOXO	forkhead box O
FYVE	Fab1p, YOTB, Vac1p and EEA1
Gab2	Grb2-associated binding protein 2
Gads	Grb2-related adaptor protein 2

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
GSK-3	glycogen synthase kinase 3
H	histamine receptor
HC	highly cytokinergic
HEV	high endothelial venules
HIFCS	heat inactivated fetal calf serum
hr	hour(s)
HRPO	horseradish peroxidase
HUVEC	human umbilical vein endothelial cells
IB-MECA	N^6-(3-iodobenzyl) adenosine-5'-N-methyluronamide, 1-Deoxy-1-[6-[((3-Iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide
IC_{50}	50% inhibitory concentration
ICAMs	intercellular cell adhesion molecules
i.d.	intradermal
i.e.	id est
IFNγ	interferon-gamma
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IKK	inhibitory κB kinase
IL	interleukin
IL-1ra	IL-1β receptor antagonist
IMDM	iscove's modified dulbecco's medium
i.p.	intraperitoneal
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
i.v.	intravenous
KC	keratinocyte-derived chemokine
K_d	dissociation constant
kDa	kilo Dalton
KI	knock in
KO	knock out
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LEAF	low-endotoxin, azide-free
LFA-1	lymphocyte function-associated antigen-1
L-Gln	L-glutamine
LPR	late-phase response
LPS	lipopolysaccharide
LT	leukotriene
mAb	monoclonal antibody
Mac-1	macrophage antigen-1
MadCAM-1	mucosal addressin cell adhesion molecule-1
MAPK	mitogen-associated protein kinase
MCs	mast cells
MC-CPA	mast cell carboxypeptidase
MCP	mast cell protease
MCP-1	monocyte chemoattractant protein-1
MC_{TC}	tryptase- and chymase-positive mast cells
MC_T	tryptase-positive mast cells
MIP-1α	macrophage inflammatory protein-1alpha
MIP-1β	macrophage inflammatory protein-1beta
MMC	mucosal mast cells
MMPs	matrix metalloproteinases
mTOR	mammalian target of rapamycin
NFκB	nuclear factor κB

NGF	nerve growth factor
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
PDGFR	platelet-derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PE	phycoerythrin-labeled
PECAM	platelet-endothelial cell adhesion molecule
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
p.o.	per os
PSA	passive systemic anaphylaxis
PSGL-1	P-selectin glycoprotein ligand-1
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
TGFβ	transforming growth factor-beta
T_H	T helper
TH	tec homology
TLR	toll-like receptor
TMT	transmembrane tryptase
TNF-α	tumor necrosis factor-alpha
TRIM	T-cell-receptor-interacting molecule
TSC	tuberous sclerosis complex
TSC1	hamartin
TSC2	tuberin
VCAM-1	vascular cell adhesion molecule-1

VLA	very late antigen
Vps	vacuolar protein sorting
vs.	versus
W	white spotting locus
WT	wild-type
YxxM	Tyr-xaa-xaa-Met
ZAP70	zeta-chain associated protein kinase 70 kDa

ABSTRACT

Allergic disease is highly prevalent in humans, and is a disorder of the immune system referred to as atopy. Allergy - or type I hypersensitivity - is characterized by excessive activation of mast cells by IgE, which results in inflammatory responses. Common allergic reactions include eczema, hives, hay fever, asthma and food allergies. Severe allergies may result in life-threatening anaphylactic responses and potentially death.

Mast cells, the primary effector cells in allergy and inflammation, are derived from circulating hemapoietic progenitor cells, mature in vascularized tissue and play important role in both innate and adaptative immunity. They express on their surface different receptors: the high affinity IgE receptor, FcεRI, activated upon IgE/antigen complexes cross-linking leading to degranulation; c-kit, the receptor for SCF important for growth and differentiation of mast cells; G-protein coupled receptors (GPCR) for chemokines; Toll-like receptors and complement receptors that can interact with pathogens. Once activated, mast cells release a variety of stored and newly synthesized inflammatory mediators such as granules-associated mediators (e.g. histamine, proteases), lipid mediators (e.g. leukotrienes and protaglandines) and cytokines and chemokines. All these inflammatory mediators lead to immune cells recruitment at the site of inflammation.

The class I phosphoinositide 3-kinase (PI3K), which produces the second messenger PtdIns(3,4,5)P_3, has been demonstrated to be essential in mast cell activation. Indeed, PI3Kγ, the only class IB PI3K, plays a role in mast cell activation via adenosine which potentiated mast cell degranulation initiated by IgE/antigen. PI3Kγ consists of a catalytic subunit p110γ, which binds to either p101 or p84 adapter subunits. In mast cells, p84 is the predominant adaptor subunit and plays a major role in signaling downstream of GPCR. PI3Kδ, a class IA PI3K, is also important in mast cell activation especially through the c-kit receptor.

Using bone marrow derived mast cells (BMMCs) from p110γ-deficient mice and catalytically inactive p110δ mice, we investigate the role of these two kinases in a step by step analysis of mast cell recruitment from blood to the inflammation site. Our investigations revealed that p110δ remains essential for signaling downstream of the c-kit receptor but

p110γ is the main isoform involved in mast cell adhesion to endothelia. This process is mediated by interaction between α4β1 integrin on mast cells and VCAM-1 expressed on endothelial cells. We found that functional p110γ is required for mast cell-derived TNF-α triggering activation of endothelia. In tissues, mast cells adhere to extracellular matrix protein like fibronectin. Activation of cell surface receptors leads to BMMCs adhesion to fibronectin in a p110γ-dependent manner and mediated by α5β1 integrin. *In vivo*, IgE-mediated mast cell recruitment as well as IgE/antigen-induced passive cutaneous anaphylaxis are severely impaired by the absence of p110γ. In addition, both p110γ isoform-selective inhibitor and Enbrel, a TNF blocker, protected mice against anaphylaxis response. Altogether this suggests a critical role for PI3Kγ and TNF-α in mast cell activation and recruitment during allergy and inflammation.

INTRODUCTION

1. Mast cells

1.1. History

Mast cells were first described by Paul Ehrlich in 1878. In his doctoral thesis, he discussed their unique staining characteristics and large granules which led him to name them "Mastzellen" in the belief that they could nourish the surrounding tissue (from the Greek masto "I feed") [1]. In 1900, Jolly demonstrated the bone marrow origin of mast cells [2]. In the 1950s, mast cell granules were discovered to be the major reservoir of histamine [3] and to participate in allergic diseases [4-6]. The identification of interleukin-3 (IL-3) as a mast cell growth factor in the 1980s enabled to culture them and facilitate their studies [7,8]. Since then, studies have addressed the role of mast cells in both innate and adaptive immunity [9,10], including their ability to phagocytose bacteria [11] and their dominant role in the Arthus Reaction (local inflammation response due to deposition of immune complexes in tissues) [12].

1.2. Origin, morphology and distribution

During the 100 years after their discovery, mast cells were believed to be a component of connective tissue derived from undifferentiated mesenchymal cells [13]. However, Kitaura and co-workers established that mast cells are derived from multipotent hematopoietic progenitors in the bone marrow. Indeed, W/W^V mice, devoid of mast cells, were able to develop mast cells if the mice received bone marrow cells from a normal animal [14,15]. Mast cells originate from pluripotent $CD34^+/c\text{-}kit^+/CD13^+$ cells in humans and from $Lin^-/Sca.1^-/Fc\varepsilon RI^-/c\text{-}kit^+$ cells in mice. Mast cells precursors circulate in the blood and migrate into vascularized tissues, where they differentiate and maturate under the influence of growth

factors and cytokines [16-20]. IL-3 and the c-kit ligand, also known as stem cell factor (SCF) are the principal cytokines that promote murine mast cell maturation and proliferation [8,21,22].

Mast cells are about 10-20 μm diameter in size. They are widely distributed in connective tissue and contain metachromatic granules composed of heparin and histamine which can be stained with dye as toluidine blue and make them readily identifiable in tissues[23].

Mast cells are preferentially located at the interface of external and internal environments such as the airways, the gut and most abundantly, the skin where they are strategically positioned to trigger allergic reactions but also to provide protective host defense responses against pathogens [24,25]. The analysis of human cutaneous mast cells shows no difference in mast cell number regarding the sex or the age of the individuals examined. Interestingly, mast cell numbers are higher in superficial skin layers and at peripheral skin sites (hand, feet, facial skin) where the risk of bacterial infection is more prominent [26].

1.3. Heterogeneity

Although mast cells share many characteristics, it has been known since their discovery that they represent a heterogenous population.

The rodent mast cell subtypes are based on phenotypical, morphological and histochemical differences: connective tissue mast cells (CTMC) are found in the skin and peritoneal cavity whereas mucosal mast cells (MMC) are located in the intestinal lamina propria [27]. MMC can rapidly expand during T cell-dependent immune responses to certain intestinal parasites [28,29]. In contrast, CTMC display little or no T cell-dependence and are present in normal numbers in athymic nude mice [30]. The differences between these two populations, summarized in **Table 1**, include size, histamine content, proteoglycans and neutral proteases composition.

Table 1: Rodent mast cell characteristics

Characteristics	Connective Tissue Mast Cells (CTMC)	Mucosal Mast Cell (MMC)
Size (μm)	10-20	5-10
Formaldehyde fixation	Resistant	Sensitive
Staining	Safranin	Alcian Blue
T-cell dependence in 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 D_2	+	+
Leukotriene C_4	-	++
Activated by		
FcεRI aggregation	Yes	Yes
Compound 48/80	Yes	No
Substance P	Yes	No
Inhibited by sodium cromoglycate	Yes	No

Table adapted from [27].

Human mast cells also exhibit differences in size, histochemical properties, quantities of stored mediators, sensitivity to stimulation and drug susceptibility. Like in rodents, human mast cells can be classified into two populations according to their neutral protease content: MC_{TC} because they contain both tryptase and chymase whereas MC_T contain only tryptase [31]. The MC_T are principally found in the alveolar septa of the lung and in the small intestinal mucosa whereas the MC_{TC} predominate in the skin and the small intestinal submucosa [32] **(Table 2)**. Therefore, the human MC_T corresponds most closely to MMC whereas the MC_{TC} corresponds closely to the rodent CTMC.

Table 2: Characteristics of human mast cell subsets

Characteristics	MC_T	MC_{TC}
Neutral protease	Tryptase	Tryptase Chymase Carboxypeptidase Cathepsin G
Granule ultrastructure	Scrolls	Lattice/grating
T-cell dependence	Yes	No
Inhibited by sodium cromoglycate	Yes	No
Distribution, %		
Skin	<1	>99
Alveolar tissue	93	7
Nasal mucosa	66	34
Tonsils	40	60
Small intestine		
Mucosa	81	19
Submucosa	23	77

Table adapted from [27].

1.4. Growth and differentiation

Normal mast cell development and survival, and therefore mast cell function, are disrupted in mice lacking functional membrane SCF (Sl/Sl^d) or with mutations within the c-kit receptor (W/W^V) with less than 1% of wt levels of skin mast cells and no detectable mast cells in the peritoneal cavity, respiratory system, gastrointestinal tract or other sites [15,33]. The mast cell-deficiency can be overcome by mast cell transplantation via adoptive transfer into the peritoneal cavity or via intradermal or intravenous injection [34]. Therefore, the major factors for mast cell growth and development include SCF, IL-3 and T_H2-associated cytokines such as IL-4, IL-9 and IL-10 [35].

Mast cell progenitors proliferate and differentiate *in vitro* in the presence of SCF and IL-3 [18]. *In vitro*, mouse bone marrow cultured in IL-3 containing media gives rise to cultures of about 85% or more mast cells after 4-5 weeks [36] whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) [37], interferon-γ (INF-γ) [38] and transforming growth factor-β (TGF-β) [39] inhibit the differentiation of IL-3-dependent mast cells. IL-3 is both necessary and sufficient for murine bone marrow-derived mast cell (BMMCs) growth *in vitro*, although more factors are required for maturation. Alone, SCF have a limited early effect on BMMCs survival, however SCF, IL-4 and IL-9 act in synergy with IL-3 to enhance mast cell proliferation [27]. Murine bone marrow cultured in presence of IL-3 and SCF gives rise to BMMCs corresponding to CTMC phenotype [40].

1.5. IgE and its high affinity receptor FcεRI

Mast cell activation is initiated upon interaction of a multivalent antigen (allergen) with its specific IgE antibody attached to the cell membrane via its high-affinity receptor, FcεRI. Cross-linkage of IgE-allergen brings the receptors into juxtaposition and initiates mast cell activation and mediator generation and release.

IgE is produced following presentation of antigen by antigen-presenting cells (APC), such as a dendritic cell (DC) or B cell, to T_H2 cells at local lymph nodes. Activated T cells then release IL-4, IL-13 and CD154 (CD40 ligand). CD154 engages CD40 expressed on APC, resulting in activation of the APC. In B cells this results in isotype class switching from IgM to antigen-specific IgE by a complex process, followed by secretion of allergen-specific IgE [41]. IgE sensitizes mast cells to antigen locally [42], thus minimizing systemic reactions and promoting survival of the mast cells [43]. Circulating levels of IgE are normally very low, in the range of 1 to 400 ng/ml (the lowest of the five human immunoglobulin isotypes) and their increase correlates with allergic disease [44].

FcεRI is a multimeric cell-surface receptor that binds the Fc fragment of IgE with high affinity [45]. It is a tetrameric protein complex consisting of an α-chain (FcεRIα), a β-chain (FcεRIβ) and a homodimer of disulfide-linked γ-chains (FcεRIγ) **(Figure 1)**. The α, β and dimeric γ chains form a complex in the plasma membrane through hydrophobic and electrostatic noncovalent interactions involving both covalently and noncovalently bound

lipids [46,47]. The α-chain belongs to the immunoglobulin superfamily and comprises a transmembrane domain, a short cytoplasmic tail and two extracellular immunoglobulin-related domains that bind a single IgE molecule, [48-51]. The β subunit has four transmembrane domains separating amino and carboxy terminal cytoplasmic tails [51,52]. The γ-chain consists essentially of a transmembrane region and a cytoplasmic tail [53]. The FcεRIβ- and γ-chains have no role in ligand binding. FcεRI, has no intrinsic tyrosine kinase activity, like other antigen receptors, but instead contains immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic tails of the β and γ chains, which get phosphorylated after antigen cross-linking of receptor-bound IgE molecules [54]. The α subunit is unique to FcεRI. However, the β and γ subunits are shared with others Fc receptors; the β-chain with the low-affinity IgG receptor, FcγRIII, and the γ-chain with both FcγRIII and the high-affinity IgG receptor, FcγRI [55-57].

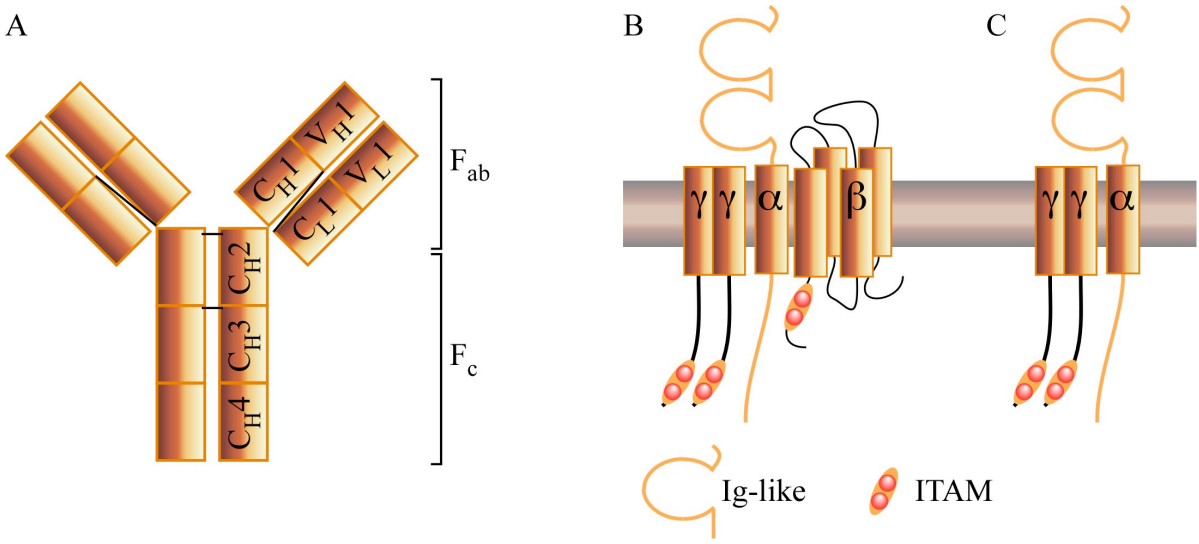


Figure 1: A. Schematic structure of IgE which contains two isotype-specific heavy chains and two light chains (H_2L_2). The F_{ab} domain contains both heavy chain and light chain components while the F_c domain is derived exclusively from the heavy chain. The black lines represent the disulfide bonds. B. The FcεRI receptor consists of one α-chain, one β-chain and two γ-chains. The α-chain comprises two Ig-like domains, a transmembrane domain and a cytoplasmic tail. The β subunit has four transmembrane domains while the γ-chain consists of a transmembrane domain and a cytoplasmic tail. The tetrameric form is expressed by mast cells and basophils. C. The trimeric form lacks the β-chain and is expressed by APCs.

In mouse, FcεRI is expressed on effector cells of anaphylaxis, i.e. mast cells and basophils as a tetramer whereas its distribution in humans also comprises monocytes, Langerhans cells, eosinophils and DCs as a trimeric form lacking the β-chain [58-62]. The expression of FcεRI on the surface of mouse mast cells occurs early in their differentiation and maturation [63], and mature mast cells can express FcεRI in excess of 10^5 per cell [64].

IgE binds to transmembrane- or solubilized-FcεRI receptors with high affinity ($K_d=10^{-10}$ M) [65]. The binding of IgE occurs via the Fc region of the immunoglobulin in a 1:1 ratio [66]. The Cε3 domain (immunoglobulin heavy chain epsilon constant domain 3) of the IgE-Fc region contains the binding site for FcεRI [67]. IgE binds to the extracellular domain of the α subunit without a conformational change [68]. Studies in both mice and humans have revealed that levels of FcεRI surface expression can be regulated by levels of IgE [69,70]. For example, IgE-deficient mice exhibit a dramatic reduction in FcεRI expression compared to wild-type mice, which is upregulated by incubation of IgE *in vitro* or by administration of IgE *in vivo* [71]. Other studies reported similar effects of IgE on the surface expression of FcεRI on basophils, monocytes and DCs [72,73]. Mechanistically, IgE-mediated FcεRI upregulation results from the stabilization of the cell surface receptors by the immunoglobulin. This stabilization stops internalization and degradation of FcεRI while maintaining basal synthesis. Inhibition of FcεRI internalization leads to the accumulation of more receptors at the cell surface. Initially, the accumulation comes from the preformed receptor pool and is insensible to the protein-synthesis inhibitor, cycloheximide. Later, when the pool of FcεRI is fully utilized, this process becomes sensitive to cycloheximide [71,74].

1.6. SCF and its receptor, c-kit

C-kit receptor, also known as CD117, is a receptor tyrosine kinase [75]. SCF, a hematopoietic growth factor, was identified as the c-kit ligand [76]. c-kit is encoded by the white spotting locus (*W*) [77] and expressed as several alternatively spliced isoforms (four isoforms in humans and two isoforms in mice) which give rise to proteins with molecular weights around 145 kDa [75]. SCF is encoded by the Steel locus (*Sl*) [21] and exists as two forms produced by alternative splicing: a soluble form and a membrane-bound form, which lacks the proteolytic site for processing into the soluble form [78].

The receptor is a type III receptor tyrosine kinase. The members of this subfamily share the same topology **(Figure 2)**: an extracellular part containing five immunoglobulin-like motifs, a transmembrane segment and a cytoplasmic kinase domain divided into proximal and distal regions by an insert sequence of variable length. SCF binds to the second and third Ig domains while the fourth motif plays a role in receptor dimerization [79]. The kinase domain, with the activation loop located in the distal kinase domain, are responsible for catalyzing the transfer of a phosphate group from ATP to the substrate [80].

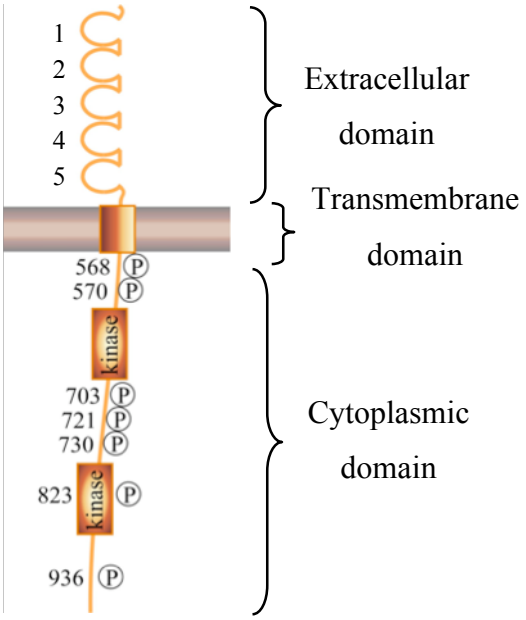


Figure 2: Structure of the c-kit receptor. Upon ligand binding, c-kit dimerizes and its intrinsic tyrosine activity is activated leading to phosphorylation of key residues. These residues constitute high-affinity binding sites for signal transduction molecules.
In the extracellular domain, 1 to 5 refer to number of the Ig-like domains. In the cytoplasmic domain, the numbers refer to the phosphorylated residues.

The receptor is expressed by hematopoietic stem cells, dendritic, erythroid, megakaryotic and myeloid progenitor cells, and pro-B and pro-T cells [81,82]. C-kit expression is lost during cells differentiation except in melanocytes, the intestinal interstitial cells of Cajal and mature mast cells [83,84]. SCF is produced by fibroblasts, endothelial cells, thymus tissue, spleen, testis and mast cells [85-89].

Although SCF has many roles in stem cell biology, its major role is related to mast cells, which are among the few cells that express c-kit after differentiation [40]. Both W/W^V and Sl/Sl^d mice with mutations in the c-kit receptor and SCF loci respectively exhibit macrocytic anemia, sterility, hypopigmentation, and are deficient in mast cells [15,33] suggesting that SCF/c-kit interaction plays an important role in the development of murine mast cells. Although W/W^V mice contain less than 1% of the number observed in wt animals, mast cell progenitors are found in the bone marrow of these mice [90]. Treatment of Sl/Sl^d mice

with SCF locally increases the number of mast cells [76]. SCF promotes survival, proliferation and maturation of mast cells *in vitro* and *in vivo* [40,91,92]. SCF also promotes mast cell degranulation by itself [93,94], chemotaxis [95] and adhesion [96]. SCF can also potentiate mast cell degranulation during IgE-dependent activation and release of histamine and arachidonic acid [93,97,98] or serotonin from mouse peritoneal mast cells [99].

1.7. Toll-like receptors (TLRs)

Consistent with their role as sentinels in host defence, mast cells have a wide variety of cell-surface receptors that can interact with pathogens. Indeed, mast cells express TLRs to respond to products of both Gram-positive and Gram-negative bacteria and participate in host defense [100-103]. The primary response to TLR ligands is the production of inflammatory cytokines such as TNF-α and IL-6 rather than degranulation [104,105]. Both rodent and human mast cells have been shown to express TLR1, 2, 3, 4, 6, 7, 8 and 9, although functional studies focused mainly on TLR2 and TLR4. The latter both synergize with FcεRI to enhance degranulation and mediator release [106]. The use of TLR2- and TLR4-deficient mice demonstrates that the response to peptidoglycans (PGN) engages TLR2 whereas response to lipopolysaccharide (LPS) is TLR4 dependent [107]. All the TLRs recognize a specific category of microbial products such as PGN by TLR2, dsRNA by TLR3 [108], LPS by TLR4, ssRNA by TLR7 and TLR8 [109] and bacterial DNA and CpG-containing DNA by TLR9 [110].

1.8. Receptors for complement components

Another mechanism to respond to pathogens is provided by the complement receptors found on the mast cell surface. Mast cells have long been recognized to interact with the complement system through complement receptor 3, CR3 (also known as CD11b/CD18), CR4 (CD11c/CD18) and the receptors for C3a (C3aR) and C5a (C5aR) [111,112]. C3a, but not C5a induces degranulation and production of the chemokines MCP-1 (monocyte chemoattractant protein-1) and RANTES [113,114]. This suggest that C3a and C5a recruit mast cells to the site of complement activation and C3a-stimulated mast cells produce chemokines which promote recruitment of APCs and T cells. Indeed, recruitment of Langerhans cells to

lymph nodes induces by PGN is not observed in mast cell- or C3a-deficient mice [115]. It has been suggested that C3a binding to CR3 inhibits FcεRI signaling in some mast cells. This process requires a sequence in C3a, distinct from the C3a receptor binding domain, which inhibits FcεRIβ subunit phosphorylation and subsequent signaling events [116,117].

1.9. Mast cell mediators

Mast cells contain or generate on appropriate stimulation a group of potent biologically active mediators that can have many different effects in inflammation, tissue remodeling, and organ function at the site of mast cell activation [27,118]. Mast cells produce three main classes of mediators: preformed granule-associated mediators; newly generated lipid mediators; and cytokines and chemokines [119,120].

1.9.1. Granule-associated mediators

Histamine is an amine known to be stored by mouse and human mast cells in intracellular granules, in a crystallin complex with proteases [3] (mouse and rat, but not human, mast cells also contain serotonin [121,122]). Histamine is formed by decarboxylation of the amino acid histidine in the Golgi of mast cells and basophils [123,124]. Mast cells from human lung, skin, lymphoid tissue and small intestine contain about 3 to 10 pg histamine/cell [125,126]. Histamine is released into the local environment after initiation of the degranulation process during allergic response. It induces contraction of airway smooth muscle, mucus secretion and increases the vascular permeability and facilitates leukocytes to access affected tissues [127]. Once secreted, histamine is metabolized rapidly within 1-2 minutes [128]. On target cells, histamine binds specific histamine receptors designated H1 to H4 [129].

Mouse and human mast cells contain variable mixtures of heparin and chondroitin sulfate proteoglycans [130,131]. These proteoglycans act as extracellular mediators and as storage matrices for other preformed mediators. Indeed, by ionic interactions, they bind histamine, neutral proteases and carboxypeptidase and they contribute to the packaging and storage of these molecules within the secretory granules [132-134]. Heparin is a potent anti-coagulant and can bind certain cytokines, chemokines and growth factors produced by mast cells

themselves [118]. Heparin also regulates the localization and the activity of mast cell proteases [135].

The major protein component stored in mast cell secretory granules are neutral proteases released during exocytosis. These enzymes are called tryptases and chymases and are tryptic and chymotryptic peptidases, respectively. All these are serine proteases of the trypsin family but differ in form, activity and expression [136]. In mice, the major mast cell proteases (designated as MCP) are the chymases: mMCP-1, -2, -4, -5 and -9; tryptases: mMCP-6, -7, -11 and mTMT, a transmembrane tryptase; and carboxypeptidase (mMC-CPA) [137,138]. Mouse mast cell proteases are differently expressed at specific tissue localization: mast cells in mouse airway and intestinal mucosa express mMCP-1 and mMCP-2, whereas skin and peritoneal mast cells express mMCP-4, -5, -6 and mMC-CPA [139-142]. As in mice, the human proteases are differentially localized according to mast cell subtypes. Indeed, human tryptase is expressed in mucosal mast cells (MC_T), whereas tryptase, chymase and carboxypeptidase are present in skin and intestinal submucosa mast cells (MC_{TC}) [32]. The proteases are stored in the secretory granules as an active form and bound to heparin and chondroitin chains making them resistant to extracellular proteases [143]. Tryptase cleaves fibrinogen, activates latent collagenases, hydrolizes some neuropeptides, may causes mucus secretion and be mitogenic [144]. It also enhances vasopermeability, inflammation and airway smooth muscle hyperreactivity [145]. On the other hand, chymase converts angiotensin I to II, cleaves extracellular matrix (ECM) proteins , i.e. laminin, type IV collagen, fibronectin (FN), stimulates mucus secretion, degrades neuropeptides and can activate the metalloproteinase-2 and IL-1β precursor [146-148]. These proteases have also various roles in tissue remodeling and cellular recruitment [149-151].

1.9.2. Lipid mediators

Mast cell activation initiates *de novo* synthesis of lipid-derived substances. Among them, the most important are the cyclooxygenase and lipoxygenase metabolites of arachidonic acid, which possess potent inflammatory activity [152]. The major product of cyclooxygenase is prostaglandin (PG) D_2, whereas lipoxygenase generates leukotrienes (LTs): LTC_4, and its derivates LTD_4 and LTE_4. Human mast cells produce LTB_4 but in lower amounts than PGD_2 and LTC_4 [153,154]. LTC_4 is a potent bronchoconstrictor and increases

vascular permeability resulting in swelling and oedema and helping recruitment of leukocytes [155]. LTB_4 promotes eosinophils and neutrophils recruitment, enhances lysosomal enzyme release and augments superoxide anion production [156-158]. PGD_2 is an inhibitor of platelet aggregation [159]. It is chemotactic for T_H2 cells, eosinophils and basophils [160]. PGD_2 induces vasodilatation and increases permeability and facilitates transendothelial migration of inflammatory cells during allergic inflammation [161,162]. PGD_2 also acts as a mediator in allergic asthma. Indeed, in PGD receptor (DP) deficient mice challenged with ovalbumin after sensitization, the level of serum IgE is increased, whereas the T_H2 cytokines and leukocytes accumulation in lung is greatly reduced [163]. Platelet-activating factor (PAF) has been detected after IgE/antigen activation of mouse BMMCs, basophils and human mast cells [164,165]. PAF aggregates, degranulates platelets and causes blood vessels to dilate [166]. It is a potent mediator which induces bronchoconstriction, systemic hypotension and can induce anaphylaxis shock and death [167]. Mice lacking the PAF receptor or treatment of PAF receptor antagonist protect animals against anaphylaxis [168,169].

1.9.3. Cytokines and chemokines

Mast cells represent a potential source of cytokines, growth factors and chemokines. The synthesis and release of these products can be IgE-dependent or not but little is known about their regulation and secretion by patient with mastocytosis.

TNF-α was the first cytokine localized in mast cells [170]. TNF-α is stored in small amounts in the secretory granules and its transcription is induced after mast cell activation via FcεRI [171]. TNF-α can also be released after LPS stimulation through TLR2 and PGN stimulation via TLR4 [101]. TGFβ is released by BMMCs after IgE/antigen stimulation and activates fibroblasts for the secretion of MCP-1 [172,173]. IL-1β is produced by BMMCs upon activation by IgE/antigen or LPS [101,174]. IL-3 affects development and survival of eosinophils, basophils and mast cells. Both human and mouse mast cells have the ability to release IL-3 in response to high-affinity IgE receptor [175,176]. IL-4 is involved in the regulation of IgE biosynthesis. Human lung mast cells rapidly released IL-4 upon IgE-dependent stimulation [177]. BMMCs stimulated with PGN also produce IL-4 [101]. IgE/antigen stimulation has been reported to induce IL-4 secretion from BMMCs when cells are treated with a combination of IL-3/IL-4/GM-CSF but not with IL-3 alone [178]. IL-5 mRNA levels is increased in mouse mast

cells and the protein is produced by human mast cells activated by FcεRI [179,180]. IL-6 stimulates the differentiation and maturation of B cells and the production of immunoglobulins. IL-6 is secreted by both human and mouse mast cells stimulated by IgE/antigen, LPS, PGN, calcium ionophore and SCF [101,181,182]. IL-8 is a potent chemotactic cytokine for neutrophils and lymphocytes [183,184]. IL-8 is released from human mast cells stimulated with phorbol 12-myristate 13-acetate (PMA) or calcium ionophore [185]. Keratinocyte-derived chemokine (KC), the functional murine homolog of IL-8, is secreted by FcεRI-activated mouse mast cells [186]. BMMCs activated by IgE/antigen or calcium ionophore are able to produce enhanced levels of IL-9 (as well as IL-3, IL-5, IL-6 and TNF-α) when costimulated with IL-1 [187]. IL-10 enhances proliferation and Ig production by B cells via the CD40 pathway [188]. It has been shown that human lung mast cells are capable of producing IL-10 in response to IgE-dependent stimulation [189]. Human and murine mast cells produce IL-13 in response to PMA, LPS, PGN, calcium ionophore, SCF or through their IgE receptor activation [190-192]. IL-13, along with IL-4, is implicated in the induction of IgE synthesis. Human mast cells contain and release IL-16 after PMA or C5a activation. This provides a possible link between mast cell activation and T cell accumulation in mast cell-dependent inflammation because IL-16 functions as a CD4+ T cell chemoattractant cytokine [193]. Human and mouse mast cells are also an important source of GM-CSF. This IgE-dependent GM-CSF release can be potentiated by SCF [194]. Nerve growth factor (NGF) is produced in both human and mouse mast cells after IgE/antigen stimulation [195]. SCF regulates mast cell differentiation and growth. Human skin and lung mast cells store and secrete SCF upon activation [89,196].

Chemokines are chemotactic cytokines regulating the migration of hemapoietic cells, including mast cells. There is also evidence that cultured mast cells and mast cell lines release chemokines such as macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MCP-1 and RANTES which can recruit effector cells during immune response [197-199].

1.10. Adhesion Molecules

The complexes forming the functional component of cell adhesion comprised three general classes of protein: the cell adhesion molecules (CAMs), the ECM proteins and the peripheral membrane proteins. There are four major families of the CAMs: the integrins, the

immunoglobulin superfamily, the selectins and the cadherins [200,201]. Among them, the integrins are the best studied as receptors of mast cells.

1.10.1 Integrins

The integrins are non-covalently linked, heterodimeric molecules containing an α and a β subunit, each with a large extracellular domain, a transmembrane domain and a short cytoplasmic tail [202,203] **(Figure 3)**.

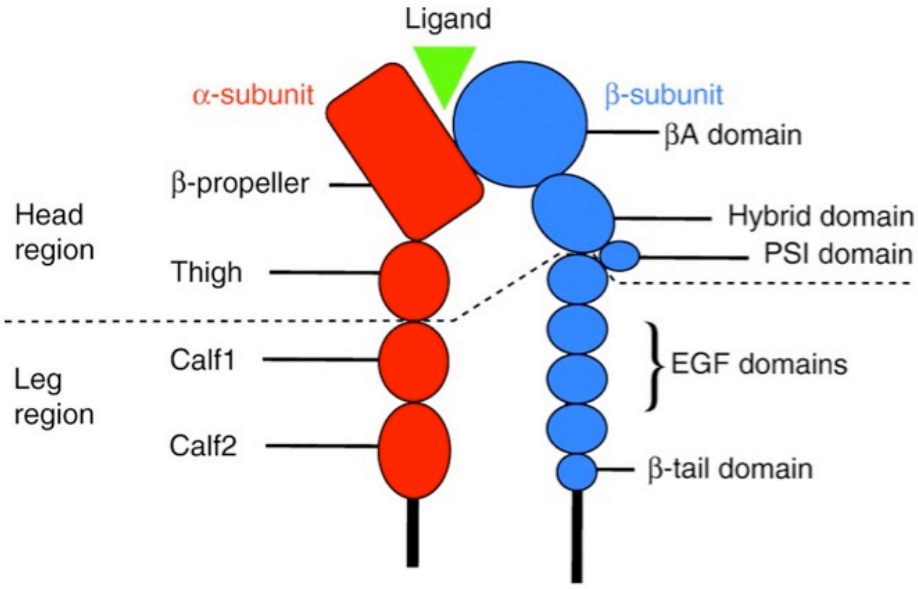


Figure 3: Schematic diagram of integrin structure. The overall structure is that of a head region (propeller and thigh domains of the α-subunit and the βA, hybrid and PSI domains of the β-subunit) supported on two legs that are made up of the calf1 and calf2 domains in the α-subunit and the EGF repeats and β-tail domain in the β-subunit. The binding of ligands takes place at an interface between the α and the β subunit [204].

These heterodimeric receptors mediate active connection between extracellular adhesion molecules and the intracellular actin cytoskeleton. In mammals, 18 α and 8 β subunits have been characterized and form 24 different receptors **(Figure 4)**. Integrin-ligand interactions provide physical support for cells in order to maintain cohesion, to enable the generation of traction forces to permit movement, and to organize signaling complexes to modulate differentiation and cell fate [205]. The signal transduction through integrins occurs in two directions: from the extracellular environment to the cytoplasm, termed "outside-in

signaling" and from the cytoplasm out to the extracellular domain of the receptor, termed "inside-out signaling" [206]. Integrin activation converts them from a low-affinity (resting) state to a high-affinity (activated) state.

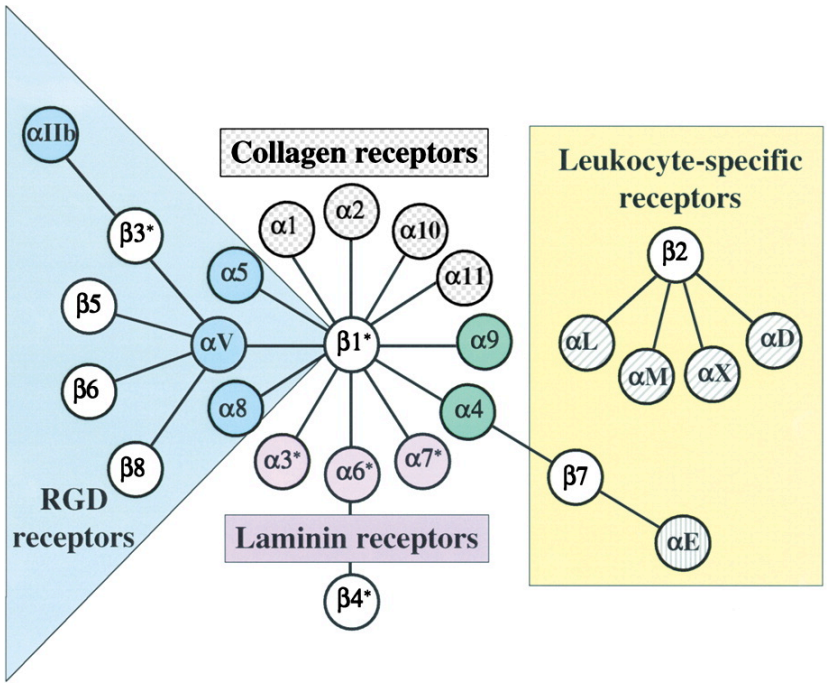


Figure 4: The Integrin Receptor Family: the mammalian subunits and their αβ associations form 24 distinct integrins. These can be considered in several subfamilies based on evolutionary relationships (coloring of α subunits), ligand specificity and, in the case of β2 and β7 integrins, restricted expression on white blood cells [207].

Human mast cells from the lung, uterus and skin have been shown to express the integrin molecules β1 (CD29), β2 (CD18), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), αIIb (CD41), αV (CD51), β3 (CD61) and β7 [52-60]. On the other hand, the integrin molecules β1, α4, α5, α6 (CD49f), αV, αIIb, β3 and β7 are expressed on the surface of BMMCs. All these integrin molecules have one or more natural ligands which are summarized in **Table 3**. During mast cell differentiation, the levels of α4β1 gradually decrease whereas the levels of α5β1 remain high [211,212].

Activated mast cells adhere to ECM proteins such as fibronectin, vitronectin, laminin, fibrinogen that bind to integrins expressed on the mast cell surface.

Table 3: The integrin family in human and murine mast cells

Integrin	Alternative names	Ligands
αIIbβ3	gpIIb/IIIa	Fibrinogen, vitronectin, fibronectin
α3β1	VLA-3, CD49c/CD29	Laminin, collagen I, fibronectin
α4β1	VLA-4, CD49d/CD29	Fibronectin, VCAM-1, MadCAM-1
α4β7		Fibronectin, VCAM-1, MadCAM-1
α5β1	VLA-5, CD49e/CD29	Fibronectin
α6β1	VLA-6, CD49f/CD29	Laminin
αVβ3	CD51/CD61	Vitronectin, fibronectin, PECAM-1, fibrinogen

Table adapted from [213].

1.10.2. Immunoglobulin superfamily (IgSF)

The IgSF is a large group of surface and soluble proteins involved in recognition, binding, or adhesion processes of cells. This superfamily includes cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules, certain cytokine receptors and intracellular muscle proteins [214,215]. In the immune system, IgSF members play a critical role in cellular adhesion. They express repeated immunoglobulin-like domains at their extracellular N-termini. These Ig domains are globular loop-like structures stabilized by sulphydryl bridging. Key members include the Intercellular Cell Adhesion Molecules (ICAMs), Vascular Cell Adhesion Molecule-1 (VCAM-1), the peripheral addressin, MadCAM-1 and the Platelet-Endothelial Cell Adhesion Molecule (PECAM). Structurally, the ICAMs on leukocytes contain two to five extracellular Ig domains [216]. ICAM-1, also known as CD54, is express on the membranes of leukocytes and endothelial cells and its level can be increased by IL-1 α and TNF-α [217,218]. ICAM-1 is a ligand for lymphocyte function-associated antigen-1, LFA-1 (αLβ2 integrin) and Mac-1 (αMβ2 integrin), receptors found on leukocytes. When activated, leukocytes bind to endothelial cells via ICAM-1/LFA-1 and then transmigrate into tissue [219]. VCAM-1, also known as CD106, contains six or seven Ig repeats and is expressed by many different cell types including activated endothelial cells, bone marrow stromal cells, spleen stroma cells, thymic epithelial cells and some DCs in the

spleen. The cytokines IL-1β, IL-4, TNF-α and IFN-γ up-regulate VCAM-1 on endothelial cell surface [218,220]. VCAM-1 is a ligand for α4β1 and α4β7 integrins [221,222]. The interaction between VCAM-1 and integrins expressed on leukocytes is involved in the extravasation of leukocytes through the endothelium to sites of inflammation [223]. MadCAM-1, containing five Ig domains, is expressed selectively on high endothelial venules (HEV) and lamina propia venules [224,225] and binds to both α4β7 integrin and L-selectin [226,227].

Human intestinal mast cells express ICAM-1 on their surface [210]. But mast cells have also been reported to induce ICAM-1, VCAM-1 and E-selectin expression on endothelial cells via their release of TNF-α [228,229]. Similarly, co-culture of mast cells with fibroblasts markedly increases ICAM-1 and VCAM-1 surface expression on fibroblasts [210,230].

1.10.3. Selectin family

Selectins are the main receptors that mediate the initial capture of circulating leukocytes to vascular endothelial surfaces followed by rolling adhesion [231]. The selectins comprise a three-member family with a highly conserved N-terminal C-type lectin and epidermal growth factor (EGF)-like tandem domains that bind sialyl-Lewis^X-like carbohydrate ligands [232]. L-selectin (CD62L) is expressed on most circulating leukocytes and initiates leukocyte capture events in high endothelial venules in secondary lymphoid tissues and at peripheral sites of inflammation. L-selectin interacts with GlyCAM-1 and CD34 [233,234]. E-selectin (CD62E) is expressed only on endothelial cells activated by cytokines such as IL-1, LPS and TNF-α [235]. P-selectin (CD62P) is stored preformed in granules of endothelial cells and in the α granules of activated platelets [236]. In response to mediators such as thrombin and histamine, P-selectin is rapidly mobilized to the plasma membrane [237]. Its primary ligand is PSGL-1 (P-selectin glycoprotein ligand-1) constitutively found on leukocytes [238]. P-selectin is largely responsible for the rolling phase of leukocyte adhesion cascade. Mast cells are known to release both histamine and TNF-α and in this respect might be important in E- and P-selectins regulation on endothelial cells. In fact, mast cells induce upregulation of P-selectin and E-selectin by secretion of histamine and TNF-α respectively on endothelia [229,239]. It has also been reported that histamine produced by mast cells induce IL-6 and IL-8 secretion by endothelial cells. This process is concentration-dependent and inhibited by H1 or H2 receptor antagonists [240].

The regulation of both CAMs and selectins on endothelial cells suggest that mast cells contribute to cell recruitment in inflammation [241].

1.11. Adhesion to Extracellular Matrix (ECM)

To understand the basis of mast cell biology, it is necessary to understand the interactions between mast cells and extracellular connective tissue matrix components. Adhesion of mast cells to components of connective tissue is important for the recruitment of mast cell progenitors from the circulation into tissue and the subsequent development, distribution, survival, priming and activation of mature mast cells [242].

The ECM is a complex network of glycoproteins and proteoglycans composed of collagens, laminins, vitronectin, fibrinogen and fibronectin (FN) which serve as substrate for integrins [243]. The ECM plays an essential role in survival, adhesion, migration and proliferation of cells.

1.11.1 Laminin

The laminins are a family of glycoproteins providing a part of the structural scaffolding of basal laminae in virtually all animals. Each laminin molecule is a heterotrimer composed of one α, one β and one γ chain subunits. There are fifteen laminin trimers formed of varying combinations of five α, three β and three γ chains. They can form independent networks, bind to other matrix macromolecules and mediate cell interaction by integrin, dystroglycan and other receptors. Through these interactions, laminins lead to cell adhesion and differentiation, cell shape and movement, and promote tissue survival [244,245].

In tissues, mast cells are often localized close to the basement membrane of endothelial cells and increase in number at sites of inflammation. This distribution led researchers to focus on the possibility that mast cells might adhere to laminin. It has been revealed that both mast cell lines and BMMCs possess functional laminin receptors. Contrary to mast cell lines which adhere spontaneously to surface coated with laminin, BMMCs had to be activated with PMA to adhere. This adherence is accompanied by cell spreading, redistribution of histamine granules and can be inhibited by antibodies to laminin and laminin receptors, α6β1 integrin [246-248]. Activation of BMMCs by the calcium ionophore A23187,

SCF or IgE/antigen also promote cell attachement to laminin [249]. Subsequently, laminin A was observed to be a chemoattractant for BMMCs after activation by A23187, PMA or IgE/antigen [250].

In human studies, skin mast cells and a mast cell line named HMC-1, adhere spontaneously on laminin [209,251] and adherence is enhanced by stimulation with SCF [252].

1.11.2. Vitronectin

Vitronectin belongs to a group of adhesive glycoproteins synthesized mainly by liver cells and plays roles in cell adhesion, differentiation, proliferation and morphogenesis [253,254]. Vitronectin circulates in the blood as a monomer where it contributes to hemostasis and fibrinolysis [255], but is converted into a multimeric form when incorporated into the ECM. Vitronectin contains an Arg-Gly-Asp (RGD) sequence constituting a binding site for integrins such as αVβ1, αVβ3, αVβ5 and αIIbβ3 [256].

Activated BMMCs were shown to adhere to vitronectin and this interaction was blocked using a RGD peptide or an antisera for αVβ3 [257]. Activation of BMMCs through αVβ3 results in enhanced tyrosine phosphorylation of focal adhesion kinase (FAK), a cytoplasmic protein tyrosine kinase involved in mitogenic and oncogenic signal transduction [258].

Human skin mast cells spontaneously adhere to vitronectin through αVβ3 [259,260], whereas the mast cell line HMC-1 required cell activation by PMA [251].

1.11.3. Fibrinogen

Fibrinogen (also called factor I) is a soluble plasma protein synthesized in the liver and is the main protein of blood coagulation system. It is a large protein and consists of two identical subunits containing three chains: α, β and γ, linked to each other by disulfide bonds. Fibrinogen contains two RGD sites for integrin interactions [261].

Mouse BMMCs stimulate with IgE, SCF or crosslinking of FcεRI adhere to fibrinogen in an integrin αIIbβ3 dependent manner. In addition, binding of BMMCs to fibrinogen enhanced proliferation, cytokine production and migration [262].

1.11.4. Fibronectin (FN)

FN is a glycoprotein composed of three different types of repeated modules: type I, II and III. The most abundant module is type III, which contains the RGD recognition sequence along with other binding sites for heparin and integrins. Fibronectin molecules can form two disulfide bridges at their carboxy-termini, producing a covalently-linked dimer **(Figure 5)**. FN exists in two forms: soluble plasma FN which is produced by hepatocytes and is secreted into the blood, and insoluble cellular FN which is the major component of ECM and is secreted by fibroblasts [263]. In tissues, FN polymerization is a cell-dependent process that requires direct interactions with integrin receptors [264]. So far, 11 different integrin heterodimers are known to be capable of binding to FN, and 4 of them, α5β1, α4β1, αVβ3, and αIIbβ3, trigger FN assembly *in vitro* [265,266].

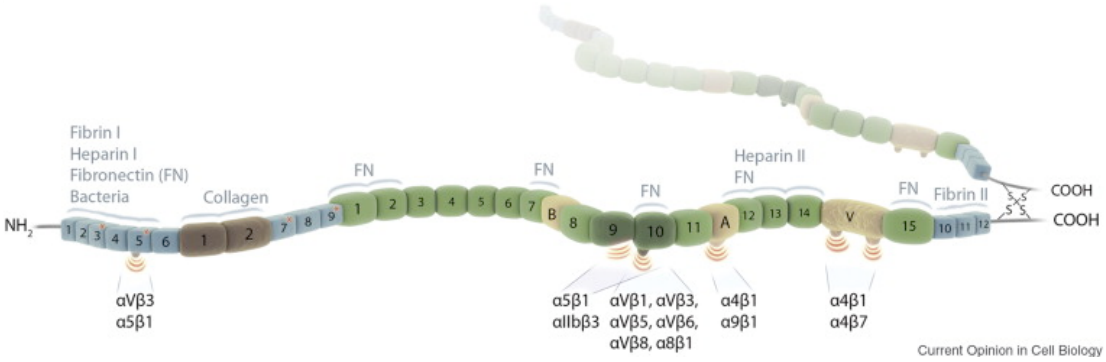


Figure 5: Scheme of the FN protein showing the repeated arrangement of the three module types (type I, blue; type II, brown; type III, green). The dimer forms via two disulfide bonds at the C-terminus. The key binding sites for integrins and other ECM protein such as heparin, fibrin and collagen are indicated. The RGD sequence is located in the 10th fragment of the type III module [267].

In the murine sytem, previous studies showed that BMMCs were able to bind to FN but required activation by PMA and that binding was dose dependent [268]. This adhesion to FN could be inhibited by an RGD-containing peptide [268]. It has also been reported that SCF stimulates BMMCs to adhere to FN at very low concentrations, *i.e.* lower than the concentration required for cell growth, suggesting that, *in vivo*, this stimulus plays an important role in mast cell recruitment [269]. Cross-linking of the high affinity IgE receptor (FcεRI) has also been reported to enhance BMMCs binding to FN [270], resulting in increased

survival [271]. It was then reported that IgE alone (*i.e.* without FcεRI crosslinking) could also increase the adhesion of mast cells to FN through the activation of both the phosphoinositide 3-kinase (PI3K) and phospholipase Cγ (PLCγ)/protein kinase C (PKC) pathways. Therefore IgE or SCF, at concentrations lower than those required for mast cell degranulation, might only play a role in mediating the recruitment, adhesion and survival of mast cells during infection or allergic reactions [272,273]. In addition, the neurotransmitter serotonin (5-hydroytryptamine (5-HT)) induces adherence of mouse BMMCs to FN and chemotaxis accompanied by actin polymerization [274]. Most of these mediators have been shown to induce α5β1-mediated adhesion to FN through a process involving increased avidity of integrin termed "inside-out" signaling [275]. Furthermore, the tetraspanin CD63, a transmembrane molecule that form complexes by lateral interactions with other tetraspanins and other molecules such as β1 integrin [276], modulates mast cell adhesion to FN [277].

Human mast cells adhere spontaneously to FN [209,251], but stimulation with SCF, PMA or A23187 enhances their adherence and provide a co-stimulatory signal for cytokine production like IL-8, IL-3 and GM-CSF [252,278]. Furthermore, IL-6 has been reported to increase, whereas TNF-α and INF-γ inhibit adhesion of human mast cell to FN [279]. Activation of human mast cells through TLR3 inhibits mast cell attachement to FN in a dose-dependent manner and results in a conformational change of β1 integrin to an inactive form [280]. More recently, IL-33, a member of the IL-1 family, and IL-1β have been shown to promote mast cell adhesion to FN, thus enhancing their survival, and to promote IL-8 and IL-13 production upon FcεRI cross-linking [281]. These findings are important to understand the mechanisms leading to tissue-specific localization of mast cells.

1.12. Adhesion to other cells

Mast cells adhere not only to ECM but also to other cells. The earliest study demonstrates that dog mastocytoma mast cells adhere to dog tracheal epithelial cells. Adhesion was abolished by pretreatment of mast cells with protease, suggesting the involvement of specific cell surface receptors [282]. Similarly, mast cells are known to adhere to fibroblasts *in vitro*. Attachment to fibroblasts is significantly impaired when mast cells do not express the extracellular domain of c-kit and likewise when fibrosblasts do not express the c-

kit ligand SCF, or by addition of a monoclonal antibody against the extracellular part of c-kit [283]. More recently, c-kit was reported to behave as an adhesion molecule and as an activator of other adhesion molecules through PI3K phosphorylation [284]. Mast cells reside in close proximity to T cells in inflamed allergic tissues and sites of infections [259]. Indeed, BMMCs and human mast cells form heterotypic aggregates with activated T cells. This cell-to-cell contact induces mast cells to release mediators like histamine and TNF-α. This mast cell-T cell interaction is mediated by ICAM-1 and LFA-1 as antibodies directed against these two adhesion molecules inhibit the attachment-induced mast cell degranulation [285,286]. Others have described that the adhesion of mast cells with endothelial cells is mediated by interactions between VCAM-1 and VLA-4 expressed on HUVECs and mast cells, respectively [210]. In addition, human mast cell progenitor express α4β1 and PSGL-1 which mediate their adhesion to activated endothelial cells under flow conditions [287].

1.13. Migration of mast cells

Mast cell progenitors enter the circulation and complete their differentiation in tissues. Significant increase of mast cell density in local tissue has been described in diseases [288-293], indicating that mast cell progenitors can be activated for migration through endothelium and also within the tissue. It has been demonstrated that SDF-1α, the only known ligand for CXCR4 expressed on human mast cells, mediates transmigration of mast cells trough HUVEC monolayers. In addition, mast cells selectively produce IL-8 in response to SDF-1α [294]. In mice, large numbers of mast cell progenitors reside in the small intestine and are constitutively recruited by a mechanism involving the α4β7 integrin [295]. In inflamed lung, α4 integrin and VCAM-1 but not MadCAM-1 are essential for mast cell progenitors recruitment [296]. Chemokine receptors expressed by mast cell progenitors and mature tissue mast cells are most likely involved in directing the progenitors from the circulation into the tissue. In CXCR2-deficient mice, the intestinal mast cells progenitor concentration is reduced indicating that the maintenance of mast cell progenitors in the small intestine is a dynamic process that requires expression of the α4β7 integrin and the chemokine receptor CXCR2 [297]. A role for CCR3 in mast cell homing has been identified in CCR3-deficient mice. In these mice, increased numbers of mast cells are found in the trachea after sensitization and allergen

challenge in an allergic airway inflammation model [298]. Migration of human lung mast cells is induced by airway smooth muscle cultures predominantly through activation of CXCR3. Importantly, CXCL10, a ligand for CXCR3, is expressed preferentially by asthmatic airway smooth muscle in bronchial biopsies and *ex vivo* cells compared with those from heathly control subjects [299]. Once in the tissue, a variety of biologic agents, including growth factors, chemokines, and adenosine nucleotides, are known to attract rodent mast cells [95,300-303]. Antigens work not only as stimulants for the release of allergic mediators from IgE-sensitized mast cells but also as chemoattractants, which can be suppressed by inhibitors of Rho-kinase/ROCK and p38 [304]. Mouse IgE molecules display a wide spectrum of heterogeneity regarding their ability to induce the production and secretion of IL-6 and TNF-α, with highly cytokinergic (HC) IgEs and poorly cytokinergic (PC) IgEs [305]. Recently, HC IgEs have been shown to efficiently activate mast cells and to promote their migration in the absence of antigen. IgE- and IgE+antigen-mediated migration involves an autocrine/paracrine secretion of soluble factors including adenosine, leukotriene B4, and several chemokines. Secretion of these factors depends on two tyrosine kinases, Lyn and Syk, and that are agonists of G-protein-coupled-receptors and signal through PI3Kγ, leading to mast cell migration [306]. In mice, mast cells are attracted by IgE and IgE-sensitized mast cells are attracted by the antigen. Therefore, IgE and antigen are implicated in mast cell accumulation at allergic tissue sites with high local IgE levels [306]. In addition to their function as mast cell activating agents, C3a and C5a have been shown to be chemotactic for human mast cells [112,307]. This chemoattraction provides a mechanism for rapid accumulation of mast cells at sites of inflammation.

1.14. Mast cell implication in disease

Mast cells are involved in both innate and acquired immunity [9,308]. Upon activation (via the FcεRI receptor or non-IgE-mediated activation through complement receptors or TLR) mast cells release a broad spectrum of preformed or newly synthesized pro-inflammatory mediators.

1.14.1. Allergic disease

Although many cells are involved in the allergic cascade, mast cells are the primary effector cells in allergic diseases due to their tissue location. IgE/antigen cross-linking to FcεRI induces degranulation and therefore release of mediators such as histamine, leukotrienes and prostaglandins which contribute to eosinophil recruitment, increase vascular permeability and smooth muscle contraction [309]. Mast cell-derived cytokines cause B cells to class switch to synthesize IgE, induce basophil histamine release, recruit neutrophils and eosinophils, and promote the development of T cells into a T_H2 phenotype [310-312]. Mast cell products induce both an immediate reaction and a late-phase response (LPR). The immediate reaction occurs within minutes thus referred as immediate hypersensitivity reaction. Late-phase reaction peaks between 6 and 12 hours following antigen challenge and is associated with cytokines/chemokines production and release in part from eosinophils, neutrophils and basophils that have entered the inflammatory site following the immediate reaction [313] **(Figure 6)**. Mast cells are also involved in chronic allergic inflammation where symptoms relapse over time.

Traditional treatment and management of allergies simply involve avoiding the allergen which is the trigger or otherwise reducing the exposure. Unfortunately, allergic persons cannot always keep away from allergen and in this respect medical treatments have been greatly improved **(Table 4)**. Pharmacotherapy comprises several antagonistic drugs used to block the action of allergic mediators or to prevent activation of cells and degranulation processes. Immunotherapy or hyposensitization is an alternative treatment, in which the patient is gradually exposed to larger doses of the allergen. This can either reduce the severity or eliminate hypersensitivity altogether [314].

Table 4: Pharmacotherapy and immunotherapy treatments used in allergy

Treatments	Drugs	Effects
Antihistamines H_1 receptor antagonists	Benadryl (diphenhydramine) Claritin (loratadine) Xyzal (levocetirizine)	Prevent the release of mediators of inflammation from human basophils and mast cells [315,316]. Target cells: endothelial cells airway smooth muscle. Relieve nasal symptoms, improve nasal airflow, reduce leukocyte infiltration (inhibit eosinophil adhesion to VCAM-1 [317]) and diminish cytokine levels [318]. Inhibition of nasal symptoms, serum total IgE and decrease IL-4 and IFNγ levels in AR [319]. Target cells: eosinophils recruited by mast cells. Inhibition of pruritus [320].
H_4 receptor antagonists	JNJ7777120	
Corticosteroids	Cortisone, Hydrocortisone, Dexamethasone	Inhibit the transcription of IL-4, IL-5 and IL-13 [321].
Epinephrine	EpiPen, Twinject	In anaphylaxis, effects on α1-adrenergic (increased vasoconstriction, peripheral vascular resistance and decreased mucosal oedema) and β2-adrenergic receptors (increased bronchodilation and decreased mediator from mast cells and basophils) [322].
Theophylline	Elixophyllin, Theochron, Uniphyl	Inhibition of PDE3 and PDE4 and antagonist of adenosine receptor. Decrease of allergen-induced migration of activated eosinophils [323].
Anti-cholinergics	Atrovent (ipratropium)	Block muscarinic receptors on airway smooth muscle and submucosal gland cells. Reduce bronchodiconstriction and mucus secretion in asthma [324].
Chromones	Nasalcrom, Intal, Opticrom (cromolyn) Tilade, Alocril (nedocromil)	Stabilize the mast cells by inhibition of Ca^{2+} mobilization, transport of Cl^-, degranulation and mediator release [325-329].
Anti-leukotrienes	Singulair (montelukast) Accolate (zafirlukast)	Block the action of cysteinyl leukotriene receptor $CysLT_1$ in the lungs and bronchial tubes [330-333].
Monoclonal anti-IgE antibody	Xolair (omalizumab)	Binds free IgE decreasing the degranulation [334,335]. Promotes FcεRI downregulation [72,334,336].
Hyposensitization (s.c. injection) and sublingual immunotherapy		Decrease in mast cell sensitivity and in IgE production by B cells. ↓ IgE/IgG4 and Th1/Th2 ratio [314].

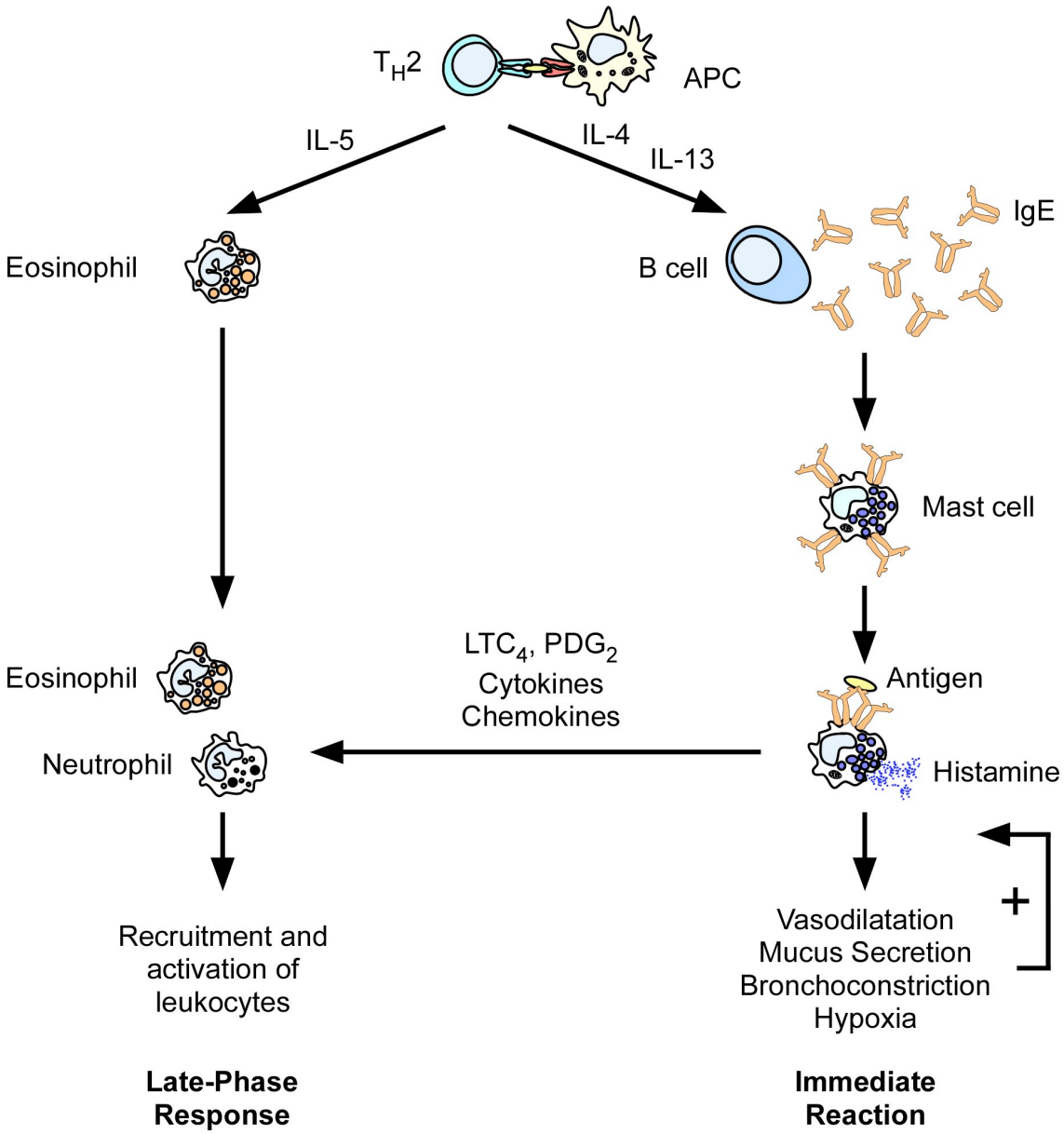


Figure 6: Mechanism of allergic cascade. IL-4 and IL-13 release from T_H2 cells results in isotype class switching from IgM to IgE in B cells. Cross-linking of IgE molecules by the antigen leads to mast cell degranulation. This immediate reaction occurs within minutes. The late-phase response (LPR), which reaches a peak between 6 and 12 hours, is associated with cytokines and chemokines production and release by mast cells and leukocytes [337].

Asthma is a complex inflammatory disorder associated with alterations in airway smooth muscle reactivity and remodeling, excessive production of mucus, bronchoconstriction and infiltration of lymphocytes, eosinophils and neutrophils. Mast cells also play an important role as several studies have reported that mast cells numbers are increased within the airway smooth muscle bundles of asthmatic patients [338-341].

Allergic rhinitis (AR) is the most common allergic disease in United States and is estimated to affect up to 40% of children and 25% of adults [342]. Mast cells constitutively reside in the nasal mucosa and do not normally go into the superficial airway epithelium. With allergen exposure, mast cells migrate to, and proliferate within, the epithelium [343]. These epithelial mast cells predominantly express tryptase and are selectively increased in AR [312,344].

Mast cells are also increased in a variety of chronic inflammation skin disorders, including atopic dermatitis (AD) [345]. Biopsies of AD lesions demonstrate an increase in mast cell numbers compared with uninvolved sites [346].

Occular allergy occurs in more than 50% of the allergic populations [309]. In symptomatic allergic patients, an increase in mast cells with evidence of degranulation is seen in conjunctival biopsies [347]. In addition to the increase in mast cells within the conjunctiva, the number of IL-4 mRNA-positive mast cells is increased threefold in seasonal allergic conjunctivitis [348].

1.14.2. Rheumatoid arthritis

Rheumatoid arthritis (RA) is the most common inflammatory disease of joints and occurs worldwide with a prevalence of about 1% in western countries [349]. If the inflammation is not controlled, the synovial tissue fills the joint cavity and finally destroys the cartilage and adjacent bone. Cytokines are involved in the inflammatory reaction. Indeed, transgenic mice that overproduce TNF-α or that lack the IL-1β receptor antagonist (IL-1ra) develop spontaneous arthritis [350,351]. The role of TNF-α and IL-1β has been demonstrated also in the K/BxN model of arthritis [352]. In the K/BxN model, infusion of autoantibodies to W/W^V mice fails to induce arthritis. In the absence of mast cells, there is no recruitment of other inflammatory cells into the joint [353]. Moreover, the number of mast cells has been shown to increase in arthritis and to correlate with the disease severity [291]. First, mast cells initiate and

perpetuate the inflammation and promote the destruction of the surrounding tissue, then they recruit and stimulate other cell types, as synovial fibroblasts and endothelial cells [354]. Mast cells localized in synovium rapidly produce and release mediators of inflammation, in particular TNF-α and IL-1β [355]. Il-1β produced by mast cells has been shown to contribute to the initiation of inflammation within joints [356]. In addition, the rapid production of LTB_4 by mast cells is important for the recruitement of CD8+ effector T cells [357,358]. Proliferation of synovial fibroblasts is a central feature of RA. The mast cell-derived tryptase has been shown to stimulate the collagen production by synovial fibroblasts [359]. Mast cell-derived TNF-α is also a potent activator of synovial fibroblasts and it stimulates their proliferation [360]. Furthermore, TNF-α induces expression of SCF in synovial fibroblasts: mast cells activate synovial fibroblasts, which in turn promote mast cell survival by secreting SCF [361]. Mast cells also contribute to cartilage and bone destruction. Mast cells can induce the expression of matrix metalloproteinases (MMPs) in synovium, and tryptase and chymase can activate latent collagenases leading to cartilage erosion and collagen degradatation[362-365]. Therefore, mast cell may represent an interesting target for future drug development.

1.14.3. Innate immunity

Beside their traditional role in allergic inflammation and RA, mast cells also act as sentinels in host defense against pathogens. Mast cells can be activated by both direct and indirect mechanisms after exposure to pathogens [366]. Direct interactions involve TLR-mediated activation and lead to cytokine, chemokine and lipid-mediator production but not necessarily degranulation [104]. Indirect activations include Fc-receptor and complement-receptor-mediated activations. Fc-receptor-mediated activation leads to the degranulation of mast cells and the production of multiple newly generated mediators [367]. Complement-receptor-mediated activation can occurs through receptors for complement components (CR3 and CR4) and receptors for complement products (C3aR and C5aR) [112]. The complement components induce the secretion of cytokines and lipid mediators as well as degranulation [368].

2. Phosphoinositide 3-kinase (PI3K)

The PI3K family of lipid kinases, catalyzes the transfer of the γ-phosphate group of ATP to the D3-hydroxy group of phosphoinositides (PtdIns). These enzymes produce various lipid products such as PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, therefore recruiting signaling molecules containing a lipid-binding domain to cellular membranes [369,370] **(Figure 7)**.

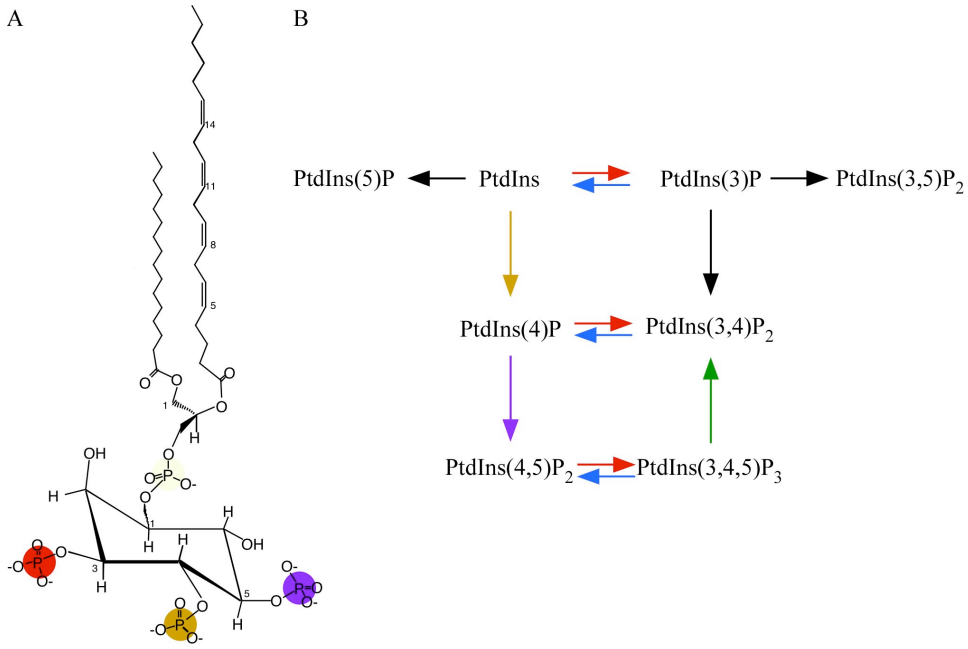


Figure 7: A. Structure of phosphatidyinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3). This structure represents the *myo*-inositol ring linked via its phosphate group to diacylglycerol. B. Metabolic reactions leading to the generation of phosphoinositide species from PtdIns. Red arrows illustrate the phosphorylation catalyzed by PI3Ks, blue arrows represent the dephosphorylation by PTEN and the green arrow, the one performed by SHIP [371].

According to their structure, lipid substrate specificity and associated regulatory subunits, the PI3K family is divided into three classes: class I, II and III [372,373] **(Figure 8)**.

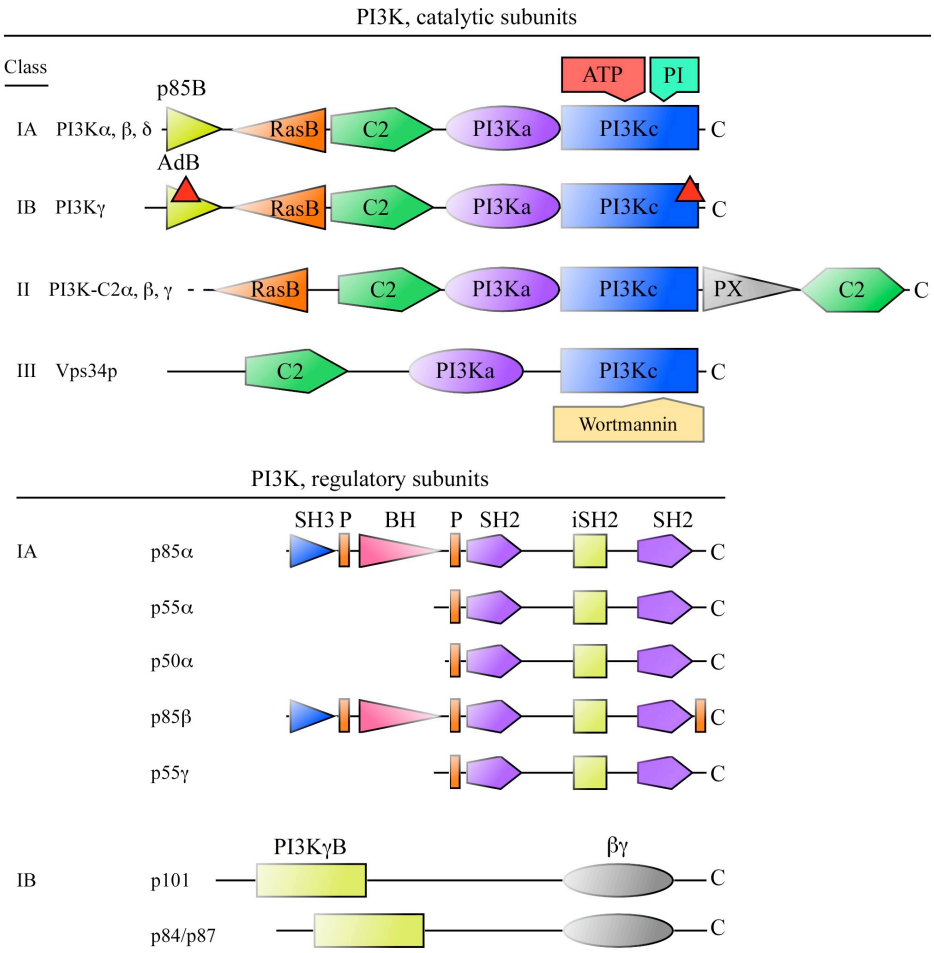


Figure 8: Structure of phosphoinositide 3-kinases. The three catalytic subunits of class IA PI3K, are composed of a p85-binding site (p85B), a Ras-binding domain (RasB), a C2 domain (protein kinase C homology domain 2), a PI3K accessory region and a catalytic core domain (PI3Kc) which interacts with ATP, phosphoinositides and the inhibitor, wortmannin. The only class IB member, PI3Kγ, has an adaptor-binding site (AdB) and two βγ interaction sites (red triangles). The class II enzymes possess no adaptor-binding site but have additional C-terminal phox (PX) and C2 domains. The class III enzymes lack both adaptor-binding site and Ras-binding domain. The regulatory subunits of class IA PI3Ks contain a proline-rich region, an iSH2 domain flanked by two SH2 domains. p85α and p85β have an additional SH3 domain and a BH domain at the N-terminus. p101 and p84/p87, the regulatory subunits of class IB PI3Ks, contain an N-terminal domain required for interaction with p110γ and a Gβγ interaction domain at the C-terminus. Modified from [374].

Class I PI3Ks are the best described of the PI3K family. Class I enzymes are the only ones able to convert PtdIns(4,5)P_2 to the second messenger, PtdIns(3,4,5)P_3 on the inner side of the plasma membrane. Although PtdIns(4,5)P_2 is their preferentiel substrate *in vivo*, they can also phosphorylate PtdIns and PtdIns(4)P *in vitro* [375,376]. Activation of class I PI3Ks by extracellular signaling involves their translocation to the plasma membrane to get access to lipid substrates. The class I PI3Ks are heterodimeric molecules composed of a catalytic subunit and a regulatory subunit, and are further subdivided into class IA and IB according to their regulatory partners and mechanisms of activation.

2.1. Class I PI3Ks

2.1.1. Structure of class IA PI3Ks

The class IA enzymes are heterodimers containing a catalytic subunit of 110 kDa: p110α, β or δ (encoded by the genes *Pik3ca*, *Pik3cb* and *Pik3cd* respectively) and one of the five regulatory p85α, p55α, p50α, p85β or p55γ subunit [377-382]. The regulatory subunits p85α, p55α and p50α are derived from a single gene, *Pik3r1*, by alternative splicing mechanisms, while p85β and p55γ are encoded by distinct genes (*Pik3r2* and *Pik3r3* respectively) [383]. Whereas the catalytic p110α and β isoforms are widely expressed in mammalian tissues, p110δ is mainly found in leukocytes [379,384]. The catalytic subunits contain an N-terminal p85-binding domain allowing them to interact with their regulatory partner, a Ras-binding domain, a C2 domain and a C-terminal kinase domain **(Figure 8)**. Each regulatory subunit has two Src Homology 2 (SH2) domains separated by a so-called inter-SH2 (iSH2) domain between the SH2 domains. The iSH2 domain interacts constitutively with the N-terminal domain of the catalytic subunit to maintain the stability of p110 in the cell. In addition, p85α and p85β possess a N-terminal Src Homology 3 (SH3) domain and a Breakpoint-cluster-region Homology (BH) domain flanked by two proline-rich regions [372]. Although no specific association has been discovered between the adaptor and catalytic subunits, p55α and p50α are more potent activators of p110 upon insulin stimulation [385,386].

2.1.2. Structure of class IB PI3Ks

The only member of the class IB enzyme identified so far is the catalytic subunit p110γ that shares structural similarities with the class IA PI3Ks [387]. The p110γ isoform appears to be present only in mammals and is abundantly expressed in white blood cells and particularly in mast cells [388,389]. The crystrallographic structure of the catalytic subunit has been reported with a C2 and catalytic domains positioned to interact with phospholipid membranes [390]. The Ras-binding domain is placed against the catalytic domain where it drives allosteric activation of the enzyme [390]. The N-terminal region contains an adaptor-binding site for the class IB regulatory subunit, p101 or the recently discovered p84/p87 [391-395]. p110γ directly interacts with Gβγ subunits via two binding sites, one in the N-terminal region and the other close to the catalytic core at the C-teminus, leading to a horseshoe conformation [396]. The crystal structures of p110γ with wortmannin, LY294002 or protein kinase inhibitors provide evidences of their interactions and conformational changes into the ATP binding pocket. These indications are then useful for the design of isoform specific inhibitors [397].

Compared to the catalytic subunit structure, little is known about the regulatory subunits that share only 30% identity mainly in the N- and C-terminal ends [392]. Both p101 and p84/p87 contain a N-terminal domain interacting with the adaptor-binding protein domain at the N-terminal of p110γ [393,398] **(Figure 8)**. The Gβγ association region is located at the C-terminal end of the regulatory subunits [393]. Moreover, the binding of the regulatory subunits to p110γ increases the activation of p110γ by Gβγ subunits [392]. The main difference between p84/p87 and p101 is that unlike p101 which is found in the nucleus, p84/p87, because it lacks the central nuclear localization signals found in p101, remains cytoplasmic [394].

2.1.3. Activation of class I PI3Ks

Signaling pathway downstream of PI3Ks class I affects cell growth, proliferation, survival as well as cell movement. Class I PI3Ks are activated by a variety of stimuli including growth factors, inflammatorymediators, hormones, neurotransmitters, immunoglobulins and antigens [374].

The class IA PI3Ks are recruited and activated downstream of phosphorylated tyrosine residues on receptor tyrosine kinases (RTK). Upon activation, the cytoplasmic tail of these receptors contains a conserved protein tyrosine kinase core that can be either autophosphorylated and/or phosphorylated by intracellular protein kinases, in particular at conserved Tyr-xaa-xaa-Met (YxxM) motifs [399]. Upon ligand binding, these receptors dimerise resulting in the phosphorylation of the YxxM sequences. These then provide docking sites for the SH2 domains of the regulatory subunit of PI3K, which brings the p110 catalytic subunit at the plasma membrane in proximity of its lipid substrates allowing the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 [400] **(Figure 9)**. Adaptor proteins such as the IRS (insulin receptor substrate) and Gab (Grb2-associated binding protein) families can also contain YxxM motifs [401]. The RTKs (EGFR; PDGFR; FGFR) exist as monomers at the plasma membrane, apart from the insulin receptor. The binding of p85 SH2 domains to receptor cytoplasmic tail cause a conformational change of the regulatory subunit, therefore reverting the inhibitory action of p85 on p110 lipid kinase activity [402-405]. The proline-rich regions and SH3 domains of p85α and p85β can facilitate additional protein-protein interactions. Indeed, p85 has been reported to bind directly to the SH3 domains of Src, Abl, Lck, Gab2, Fyn and Lyn and p85 itself via its proline-rich motifs [406-410]. Additionally, the SH3 domain of p85 interact with proline-rich domains of dynamin GTPase, Shc (Src-homologue and collagen-homologue) and Cbl (Casitas B-lineage lymphoma) [411-413]. p85, via its BH domain, has been reported to interact with members of the Rho/Rac/Cdc42 family of small GTP-binding proteins [414,415]. All class IA PI3Ks have been reported to possess an intrinsic protein kinase activity by which the catalytic subunit can phosphorylate specific Ser/Thr residues on the adaptor subunit and autophosphorylate itself [416,417].

In immune cells, the B cell receptor (BCR) and T cell receptor (TCR) recognize antigen and mediate signal transduction through PI3K class I activation. In B cells, the cytoplasmic tail of CD19 (a B cell-specific costimulatory receptor), BCAP (B-cell PI3K adaptor protein) and Cbl exhibit YxxM motifs that, upon phosphorylation, can associate with the SH2 domains of the regulatory subunits of class IA PI3K [418,419]. In T cells, YxxM motifs are found in the cytoplasmic domains of the T cell costimulatory receptor CD28 and T-cell-receptor-interacting molecule (TRIM) [420,421]. Moreover, both BCR and TCR harbor ITAMs (immunoreceptor tyrosine-based activation motifs) in their cytoplasmic tails. Upon activation, these motifs are phosphorylated by Src-family tyrosine kinases leading to the

recruitment and activation of the SH2 domain-containing tyrosine kinase Syk, or the related kinase ZAP-70 in T cells [422,423]. Thus class IA PI3K is also activated by antigen receptor engagement and its lipid products regulate a number of downstream events.

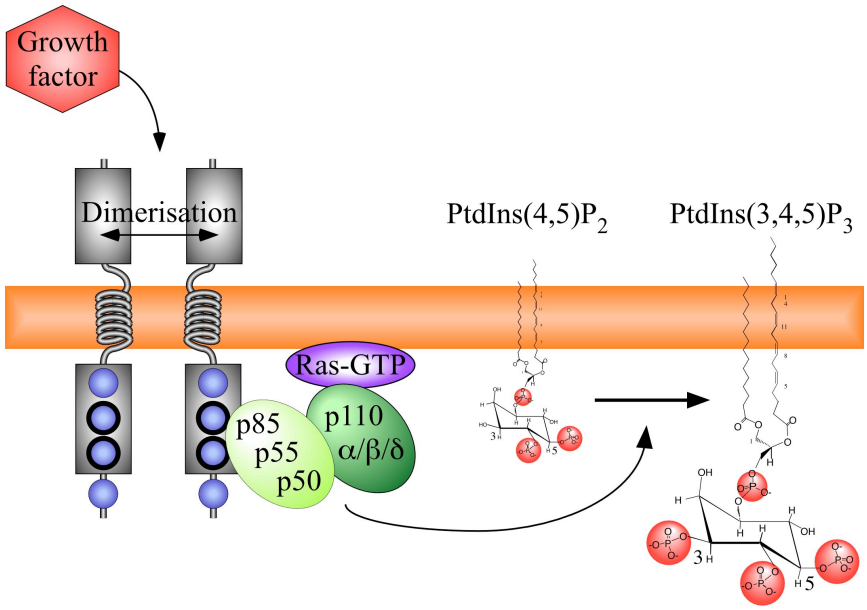


Figure 9: Activation of class IA PI3Ks. Binding of growth factors induces receptor dimerisation and autophosphorylation or phosphorylation of multiple tyrosines (blue circle), which can be located in YxxM motifs (black circles). These create docking sites for the SH2 domains of the p85, p55 and p50 regulatory subunits of class IA PI3Ks, leading to their recruitment in the signaling complex. GTP-Ras is known to bind the catalytic subunit of class I PI3Ks and to play a role in the synergistic activation of their lipid kinase activity [374,424,425].

Activation of class IB PI3K occurs downstream of G-protein coupled receptors (GPCRs), a large family of receptors proteins that includes adenosine, lipid mediators of inflammation and chemokine receptors. GPCRs are usually coupled to heterotrimeric G proteins composed of α, β and γ subunits that are associated in the inactive, GDP-bound state [387]. Activation of GPCRs upon ligand binding induced a conformational change in the receptor resulting in a decreased affinity of Gα for GDP and an increased affinity for GTP. This exchange triggers the dissociation of the Gα-GTP from the Gβγ dimer and the receptor [426]. This induces the recruitment of PI3Kγ at the plasma membrane [427]. Free Gβγ subunits interact with p110γ and the adaptor proteins p101 and p84/p87, thus activating the class IB PI3K and production of PtdIns(3,4,5)P_3 [396,428,429] **(Figure 10)**. This activation has been

reported to be dependent on the regulatory subunit [391]. PI3Kγ has also been shown to be activated by GTP-Ras (N, K, H and R) *in vitro* and *in vivo*. Indeed, crystal structure reveals a direct interaction of Ras which triggers a conformational change of the catalytic subunit p110γ. Five residues in p110γ have been shown to be critical for binding to GTP-Ras. In the so-called p110γ DASAA mutant (T232D, K251A, K254S, K255A and K256A) these essential amino acids are mutated and it can neither bind nor be activated by GTP-Ras [430]. The simultaneous analysis of $p101^{-/-}$ and p110γ DASAA suggests that Ras and Gβγ can synergistically activate PI3Kγ but differently regulate distinct PI3K effectors [431].

In addition to its lipid kinase activity, PI3Kγ also possesses an intrinsic Ser/Thr protein kinase activity that is sufficient to induce mitogen-activated protein kinase (MAPK) activation [432]. Moreover, p110γ autophosphorylates itself on Ser1101 and also transphosphorylates the p101 adapter [433-435].

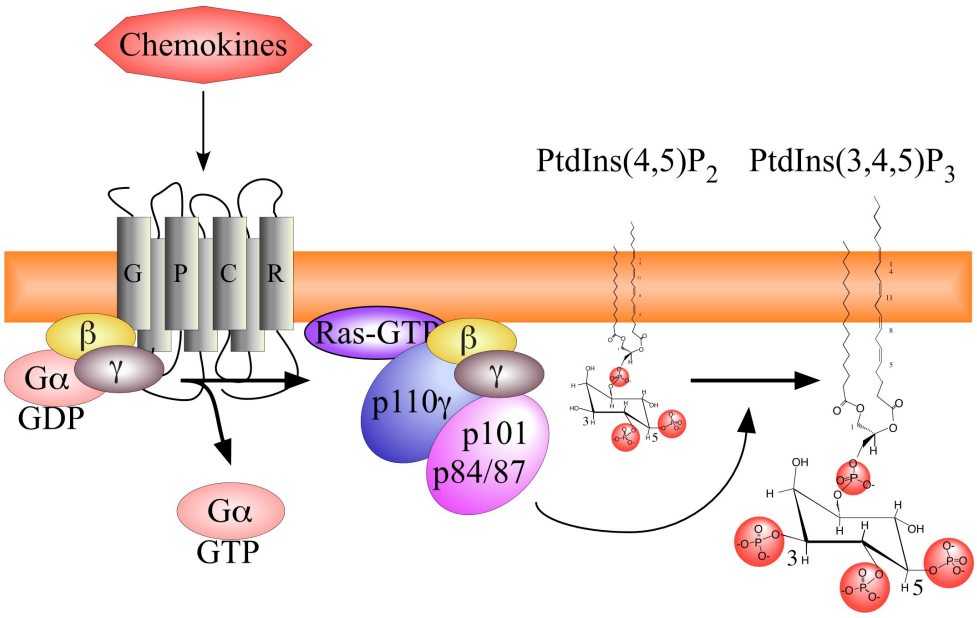


Figure 10: Activation of class IB PI3K. Binding of chemokines to GPCRs induces dissociation of the Gα-GTP from Gβγ dimer. The latter interacts with p110γ and the p101 or p84/p87 adaptor proteins. Ras-GTP is known to bind directly to and activate p110γ. Modified from [374].

Activation of class I PI3Ks leads to production of PtdIns(3,4,5)P_3 which recruits proteins with pleckstrin homology (PH) domains, *e.g.* the serine/threonine protein kinase B (PKB/Akt) **(Figure 11)**. Once PKB is recruited to the receptor-signaling complex, phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop of PKB at Thr308 [436]. Full activation of PKB required a second phosphorylation by so-called PDK2 on the hydrophobic motif at Ser 473 of PKB. The mTOR (mammalian target of rapamycin) complex 2 (mTORC2) and DNA-PKcs have been determined as PDK2 kinases [437-439]. Upon activation, PKB phosphorylates many proteins and thus positively or negatively regulates their activity. These targeted molecules can be involved in cell cycle progression, such as forkhead box O (FOXOs), cell survival via the regulation of inhibitory κB kinase (IKK) and caspase 9, or translation and transcription controlled by mTORC1 [440-442].

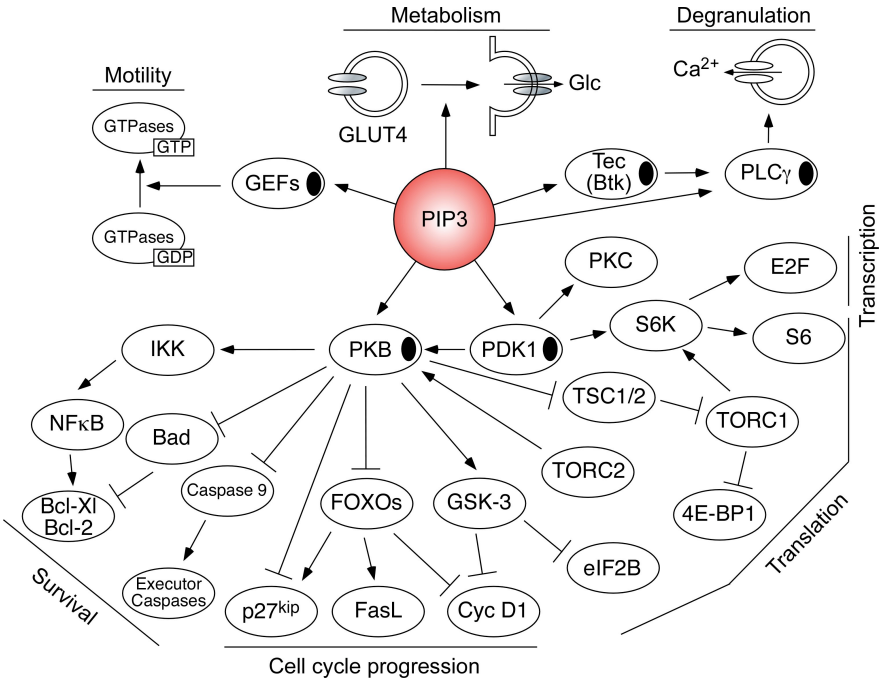


Figure 11: Central role of the PI3K pathway. PtdIns(3,4,5)P3 is produced by class I PI3Ks, and recruits proteins with PH domain (black oval). Abbreviations: 4E-BP1, eukaryotic initiation factor 4F binding protein; Bad, Bcl2-antagonist of cell death; Bcl-Xl/Bcl-2, Bcl-Xl/Bcl-2-antagonist causing cell death; Cyc D1, cyclin D1; eIF2B, eukaryotic initiation factor 2B; FasL, Fas ligand; GSK-3, glycogen synthase kinase 3; NFκB, nuclear factor κB; S6K, S6 kinase; TSC, tuberous sclerosis complex; TSC1, hamartin; TSC2, tuberin. Modified from [374].

PtdIns(3,4,5)P_3 also binds guanine exchange factor (GEFs) via their PH domains and activates them. This leads to exchange of GDP to GTP on small GTPases such as Rac, Rho and Cdc42, therefore stimulating cell motility [443,444].

The members of the non-receptor tyrosine kinases (NRTK) Tec family contain a PH domain, a Tec homology (TH) domain as well as SH2 and SH3 domains [445]. PtdIns(3,4,5)P_3 produced by activation of class I PI3Ks leads to the translocation of Tec family members at the plasma membrane bringing them in close proximity to Src tyrosine kinases which phosphorylate Tec kinases [446]. The Tec substrate, PLCγ, is activated and PtdIns(4,5)P_2 is thus hydrolyzed at the cell surface into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). This second messengers mediate the elevation in intracellular calcium and activation of PKC [447].

2.1.4. Termination of the PI3K signaling: the phosphoinositide phosphatases

As described above, the PI3Ks can be activated by a variety of extracellular stimuli and this signaling pathway has to be regulated. This is mainly controlled by two phosphoinositide phosphatases: the phosphatase and tensin homologue deleted on chromosome 10 (PTEN/MMAC/TEP1) and the SH2-containing inositol 5-phosphatase (SHIP).

The role of PTEN is to keep the levels of PtdIns(3,4,5)P_3 low by cleavage of the 3' phosphate on the inositol ring of PtdIns(3,4,5)P_3 [448]. Loss of PTEN function results in an increased concentration of PtdIns(3,4,5)P_3 and in PKB hyperactivation, confering protection from apoptotic stimuli [449]. PTEN was identified as a tumor suppressor gene located on chromosome 10 [450-452]. Frequent mutations or deletions in the PTEN gene have been found in gliomas, endometrial, prostate and breast cancers and malignant melanomas [453-457]. Germline mutations in PTEN are responsible for Cowden disease, Lhermitte-Duclos disease and the Bannayan-Zonana syndrome [458-460]. The homozygous deletion of PTEN results in embryonic lethality and analysis of the $PTEN^{-/-}$ embryonic stem cells demonstrate its crucial role in tumor suppression by controlling cellular differentiation and anchorage-independent growth [461]. The PTEN structure reveals a phosphatase domain and a C2 domain. PTEN translocates to the membrane where the C2 domain binds PtdIns(3,4,5)P_3 [462]. Since PTEN mutations are associated with tumor progression and metastasis, a role for PTEN in cell adhesion and migration has been studied. In a glioblastoma cell line, PTEN reintroduction

dephosphorylates FAK leading to inhibition of integrin-mediated cell spreading and migration [463]. In fibroblasts, PTEN deficiency leads to increased cell motility and endogenous activition of Rac1 and Cdc42, two small GTPases with well-established roles in cell motility [464]. In *Dictyostelium* and mammalian leukocytes, chemoattractants induce a pool of activated Cdc42 at the leading edge of the cell whereas PTEN is located at the posterior part [465]. In drosophila, PTEN has been shown to regulates cell size, cell number and organ size [466-468]. Heterozygous mice mutated for PTEN develop lethal polyclonal autoimmune disorders as well as impaired Fas-mediated apoptosis [469]. Furthermore, mice with T cell-specific deletion of PTEN develop lymphomas due to defect in negative selection of T cells [470].

SHIP is another phosphoinositide phosphatase family that plays important roles in negative regulation of intracellular signaling in cells. Two members of the SHIP family have been identified: SHIP-1 and SHIP-2 encoded by different genes. Multiple forms of SHIP-1 resulting from alternative mRNA splicing, protein degradation and posttranslational modification have been reported [471,472]. SHIP-1 is a 145 kDa protein and its expression seems to be restricted to haematopoitic cells, whereas SHIP-2 is a 150 kDa protein that seems to be more ubiquitously expressed [473,474]. SHIP family members are composed of a N-terminal SH2 domain, a central inositol 5-phosphatase catalytic domain, C-terminal tyrosine residues (within NPXY motifs) and proline-rich regions [475]. SHIP's N-terminal SH2 domain can interact with tyrosine phosphorylated proteins, such as immunoreceptor tyrosine-based inhibiting motifs (ITIMs) of the FcγRIIB1, and gp49B1 or ITAMs of FcεRI on mast cells [476]. Upon phosphorylation, the C-terminal tyrosine residues serve as docking site for SH2 domain-containing proteins or for the phosphotyrosine binding domain of the adaptor Shc [479]. The p85 regulatory subunit of PI3K has also been shown to interact with SHIP via the SH2 domain of p85 and the phosphotyrosines of SHIP [480]. The proline-rich regions provide binding sites for SH3 domain-containing proteins such as Grb2, Src and c-Abl [481]. Hence, these structural domains are able to support the relocalization of SHIP from the cytosol to the plasma membrane, where its catalytic activity regulates PtdIns(3,4,5)P_3 accumulation [482]. Indeed, the central catalytic domain of SHIP dephosphorylates the D-5 position on the inositol ring of PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4 [483-485]. SHIP-1, through dephosphrylation of PtdIns(3,4,5)P_3, prevents the membrane recruitment of Btk to PtdIns(3,4,5)P_3 via its PH domain and thus the activation of PLCγ [486]. SHIP-1 therefore negatively regulates the calcium fluxes. SHIP-1 also prevents the recruitment and activation of PKD1, and subsequent

activation of PKB and MAPK [487,488]. SHIP-1-deficient mice are viable but have a shorter life span due to an infiltration of macrophages and neutrophils in the lungs. They also present increased numbers of myeloid cell progenitors in both the bone marrow and the spleen. The bone marrow have decreased number of lymphoid and erythroid cell progenitors [489]. SHIP-1 also plays an important role in the regulation of B cell differentiation and function. Indeed, upon stimulation through the FcgRIIB1/BCR engagement, the SHIP-$1^{-/-}$ B cells display an increased proliferation, a prolonged Ca^{2+} influx and an enhanced MAPK activation [490]. In mast cells, SHIP-1 has been described as a "gatekeeper" of degranulation by maintaining the level of PtdIns(3,4,5)P_3 below the threshold for full mast cell activation. Indeed, SHIP-$1^{-/-}$ mast cells exhibit a more pronounced degranulation and IL-6 production as well as an increased Ca^{2+} influx and MAPK phosphorylation upon stimulation through FcεRI [491,492].
PTEN and SHIP provide tight regulation of the PI3K pathway and are essential not only for normal immune system development but also for prevention of immunopathologies.

2.2. Class II PI3Ks

Class II PI3Ks are composed of three members: PI3K-C2α, PI3K-C2β which are widely expressed in mammalian tissues [493,494] and PI3K-C2γ, restricted to few tissues including liver, breast and prostate [495,496]. Members of this class are monomers of 170-210 kDa in size. Structurally, the class II PI3Ks display homologies with the class I enzymes but lack the p85 binding motif and have different N-termini. They also possess an extended C-terminus with additional regulatory domains including the phox homology (PX) and C2 domains [497]. Pharmacological distinction can also be made. Indeed, PI3K-C2α is very resistant to the classical PI3K inhibitors wortmannin and LY294002 compared to class I members [494]. The β isoform appears to be very sensitive to wortmannin but quite resistant to LY294002 [498,499]. Class II enzymes have not yet been reported to associate with a regulatory subunit. *In vitro*, class II PI3Ks phosphorylate PtdIns to PtdIns(3)P and PtdIns(4)P to PtdIns(3,4)P_2, but unlike class I PI3Ks, not PtdIns(4,5)P_2 (although PI3K-C2α was observed to phosphorylate PtdIns(4,5)P_2 only in the presence of phosphatidylserine [494]). There is no clear indication of their *in vivo* lipid substrate, but the strong preference for PtdIns *in vitro* suggests that PtdIns(3)P is the main lipid product *in vivo* [498,500-502]. Moreover, recent studies

have reported evidences indicating that pools of PtdIns(3)P can be specifically generated upon cellular stimulation and act as intracellular second messengers [499,503,504]. The lack of an aspartic acid residue in the C2 domain prevents PI3K-C2 to function in a Ca^{2+}-dependent manner regarding phospholipid binding [500]. Deletion of the C2 domain increased the lipid kinase activity, therefore suggesting that it functions as a negative regulator of the catalytic domain [498]. Endogenous PI3K-C2α is constitutively associated with phospholipid membranes and is clearly concentrated at the trans-Golgi network. Neither the PX domain nor C-terminal C2 domains are required for this cellular localization [505]. PI3K-C2α has also been reported to bind directly to clathrin via its N-terminal region and to stimulate PI3K-C2α activity toward phosphorylated inositide substrates [506]. In addition, PI3K-C2α expression disrupts clathrin distribution and blocks clathrin-mediated endocytosis and sorting [506]. A large variety of stimuli has been found to activate class II PI3K indicating that these enzymes can regulate many intracellular processes. Insulin has been reported to activate PI3K-C2α by inducing phosphorylation of the kinase. Similarly, PI3K-C2β activity is also increased dowmstream of the insulin receptor [507,508]. Furthermore, the chemokine MCP-1 induces activation of PI3K-C2α, although the kinetics of activation are slower compared to class I enzymes [509]. Both PI3K-C2α and C2β associate with polypeptide growth factor receptors such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) indicating that these enzymes may have a role in such signaling pathways [510]. LPA stimulates PI3K-C2β, and the resulting pool of PtdIns(3)P at the plasma membrane is involved in cell migration [499]. Additionally, PI3K-C2β has also been reported to associate with the SCF receptor [511].

2.3. Class III PI3Ks

Vps34 (vacuolar protein sorting 34) is classified as the sole class III PI3K. It was discovered in a screen for mutants defective in vacuolar protein sorting in *Saccharomyces cerevisiae* [512,513]. Homologous proteins have been identified in unicellular organisms, plants, *Drosophila melanogaster*, *Caenorhabditis elegans* as well as mammals [514-519]. Class III PI3Ks are highly conserved in eukaryotes and represent the most ancient PI3Ks [520]. In mammals, hVps34 is ubiquitously expressed [515]. Class III enzymes utilize as solely substrate

PtdIns and thus produce only PtdIns(3)P, and are extremely insensitive to PI3K inhibitors such as wortmannin [521]. PtdIns(3)P binds FYVE (Fab1p, YOTB, Vac1p and EEA1) and PX domain -containing effectors. Vps34 exhibits homology with the catalytic subunit of other PI3Ks, particularly at the level of domain organization **(Figure 7)**. Vps34 is associated with a regulatory subunit Vps15, which possess intrinsic serine/threonine kinase activity [522] and corresponds to p150 in humans [523]. Vps15 kinase activity is required for activation and recruitment of Vps34 to Golgi membranes, as inactivation of Vps15 kinase activity inhibits the Vps15-Vps34 interaction and subsequently stimulation of Vps34 kinase activity [524]. Vsp34 kinase activity plays a direct role in vesicular transport from the Golgi to vacuoles, as a temperature-sensitive mutant exhibits defect in both protein sorting to vacuoles and kinase activity [524]. The human homologs of Vps34 and Vps15 appear to regulate membrane trafficking to lysosomes in a similar manner [525,526]. In early endosomes, PtdIns(3)P is formed by the hVps34/p150 complex which is recruited by the small GTPase Rab5 [527]. Early-endosomal autoantigen 1 (EEA1) is also recruited via the interaction with PtdIns(3)P and Rab5 [528]. More recently, it has been reported that hVps34 and p150 interact with Rab7 in late endosomes [529] **(Figure 12)**. Experiments in *S. cerevisiae* have provided a direct link between trimeric G-protein and Vps34 which interacts directly with a Gα subunit promoting increase of PtdIns(3)P production at the endosome [530]. The class III PI3K is required for autophagy through production of PtdIns(3)P via the autophagy-specific complex composed of Beclin-1, Vps15 and hVps34 [531] (in yeast, an additional accessory protein Vps14 is required [532]). Recent studies have reported that hVps34 also contributes to the regulation of mTOR by nutrients, since hVps34 knockdown blocks both insulin and amino acid stimulations of S6K1 [533,534].

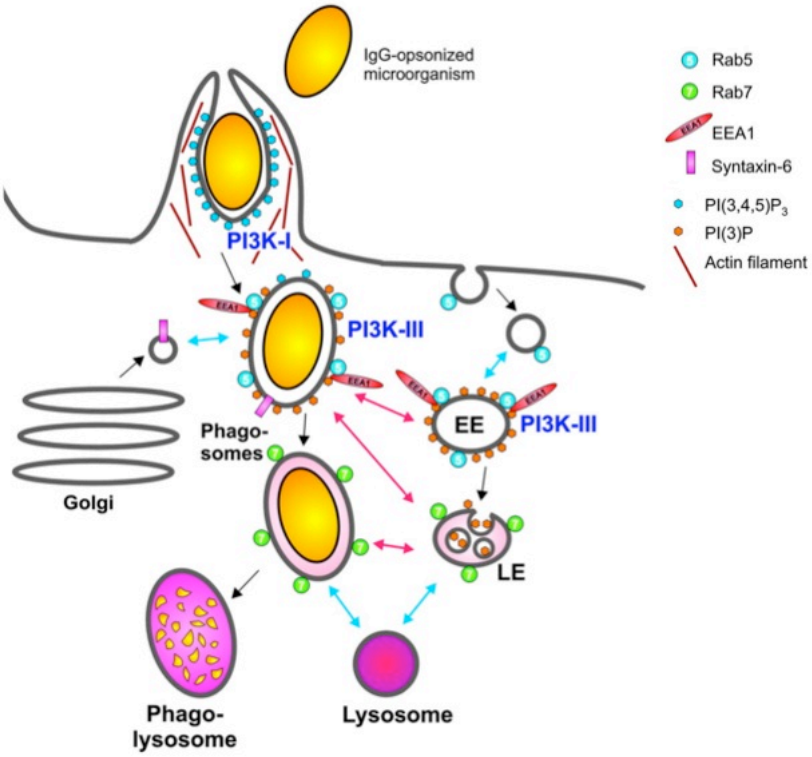


Figure 12: Class I and III PI3Ks are involved in phagocytosis. The opsonized microorganisms bind to Fcγ-receptors in the membrane of the phagocyte. This causes phosphorylation of the cytoplasmic part of the receptor and subsequent recruitment of a class I PI3K leading to PtdIns(3,4,5)P_3 production, actin rearrangements and phagosome formation. Once the phagosome is sealed, PtdIns(3,4,5)P_3 is dephosphorylated and PtdIns(3)P is formed by class III PI3K which is recruited to the early phagosome by Rab5. EEA1 is also recruited by interaction with PtdIns(3)P and Rab5. The phagosome gradually matures, eventually fuses with a lysosome forming a phagolysosome in which degradation of the microorganism ensues [535].

2.4. Targeting PI3Ks

2.4.1. Genetic targeting of class I PI3Ks

In the last few years, the development of several mouse models which are deficient for one or more regulatory or catalytic subunits of class I PI3Ks has been described [536]. Deletion of class IA PI3K regulatory subunits was reported in four mouse models: one lost the expression of all *Pik3r1* gene products thus deleting p85α, p55α and p50α and is perinatally lethal [537]; the second lost the expression of p85α but retained the expression of

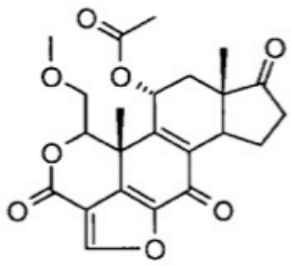
p55α and p50α [538]; the third is no longer expressing p55α and p50α but retains expression of p85α [539]; and in the fourth mouse model, the gene encoding p85β (*Pik3r2*) is deleted [540]. Deletion of the *Pik3r1* gene products leads to severe reduction in the expression of all p110 isoforms [541]. On the other hand, deletion of p85β does not affect the expression of the other class IA PI3K subunits [540]. *In vitro* and *in vivo*, PI3Ks exhibit a positive role in insulin signaling [542]. Surprisingly, all class IA regulatory subunit knock out (KO) mice display increased insulin sensitivity and improved glucose tolerance *in vivo* [540,543]. Disruption of p85α leads to impaired B cell development and functions. B cells from p85α KO have defects in proliferative responses to the polyclonal B cell activators anti-IgM, LPS and CD40 [537,538]. Moreover, p85α-deficient B cells show that BCR-induced Ca^{2+} flux and phosphorylation of IκBα as well as PKB are reduced [544]. T cell functions are intact in the absence of p85α. However, T cell lacking p85β show enhanced proliferation and survival following antigen-receptor stimulation, suggesting that p85β limits T cell expansion [545].

Compared with deletion of class IA regulatory subunits, disruption of the p110 subunits is a more direct approach to identify the function of the individual catalytic isoforms. All class I catalytic subunits have been genetically inactivated. Deletions either in *Pik3ca* or *Pik3cb* are embryonically lethal [546,547]. But recently, two groups were able to obtain viable p110β-deficient mice: the first used Cre recombinase leading to the deletion of exons 21 and 22 of the kinase domain of p110β and showed that the p110β isoform can also signals downstream of GPCR along with p110γ [548]. The second inserted a mutation in the *Pik3cb* gene (K805R), leading to the production of a catalytically inactive form of p110β. These mutants reveal that p110β is required for insulin signaling and that these mice are protected from tumor development [549]. Mice lacking p110δ or expressing a p110δ catalytically inactive have been generated and are viable. Antigen receptor signaling in B and T cells is impaired and immune responses *in vivo* are attenuated in p110δ mutant mice [550-552]. Animals lacking p110δ catalytic subunit present evidence that this isoform participates in neutrophil migration to chemoattractant but also modulates the up-regulation of selectin on endothelium [553]. The only class IB isoform, p110γ, has also been targeted either by disruption of the *Pik3cg* gene ($p110\gamma^{-/-}$) [389,554] or by generating animals with a catalytically inactive PI3Kγ (mutation of the Lys833 into Arg; $p110\gamma^{KR/KR}$) [555,556]. The chemotactic responses are impaired in neutrophils, macrophages and eosinophils from p110γ mutant mice [554,557]. The T cell survival and

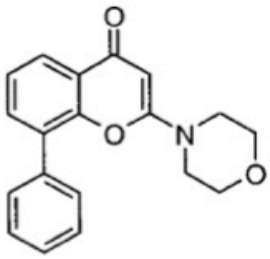
proliferation are both reduced in p110γ$^{-/-}$ mice compared to wt mice. PI3Kγ-deficient neutrophils exhibit defects in GPCR-stimulated respiratory burst [555]. It has been reported that PI3Kγ activity in vascular endothelium is also essential for neutrophil recruitment, as its activity is required for efficient selectin-mediated adhesion [558].

2.4.2. Pharmacological inhibition of the PI3K signaling

As PI3Ks are key molecules in signaling pathways, the interest in developing inhibitors as potential therapeutic agents has arised. Wortmannin and LY294002 are broad pan PI3K inhibitors. Wortmanin is the more potent inhibitor of the two with an *in vitro* IC_{50} (50% inhibitory concentration) around 5nM, whereas LY294002 is less active with an IC_{50} around 1μM [559,560] **(Figure 13)**. Both inhibitors target the ATP binding site of PI3K **(Figure 8)** which is located between a cleft formed by the N- and the C-terminal lobes of the catalytic domain [397]. Wortmannin irreversibly inhibits PI3Ks covalently interacting with a lysine residue in the ATP-binding pocket of PI3K (Lys802 in p110α and Lys833 in p110γ [397,433,561]). LY294002, in constrast, is a competitive inhibitor of the ATP site [560]. Both compounds target not only PI3K but also other kinases such as DNA-PKcs, mTOR, PI4K and ATM [562-565].



Wortmannin



LY294002

Figure 13: Chemical structures of the broad PI3K inhibitors, wortmannin and LY294002

The first synthetic generation of PI3K inhibitors have increased selectivity in respect to wortmannin and LY294002. Indeed, the compound IC87114 has been described as an ATP-competitive inhibitor able to selectively inhibit PI3Kδ activity **(Figure 14)**. The IC_{50} of IC87114 for PI3Kδ inhibition is 0.5μM whereas the values for PI3Kα, PI3Kβ and PI3Kγ are

100, 75 and 29 μM, respectively. This compound has been used to explore the importance of PI3Kδ in neutrophil migration, and TNF1α-stimulated elastase exocytosis from neutrophils in a mouse model of inflammation [566,567]. The selective inhibition of PI3Kδ has also been reported to play a role in the pathophysiology of acute myeloid leukemia [568,569]. In addition, the pharmacological inactivation of PI3Kδ in BMMCs leads to defective SCF-mediated *in vitro* proliferation and signaling, to impaired allergen-IgE-induced degranulation, and protects mice against anaphylactic allergic responses [570,571].

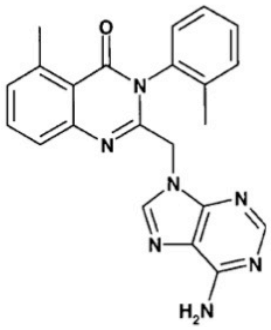
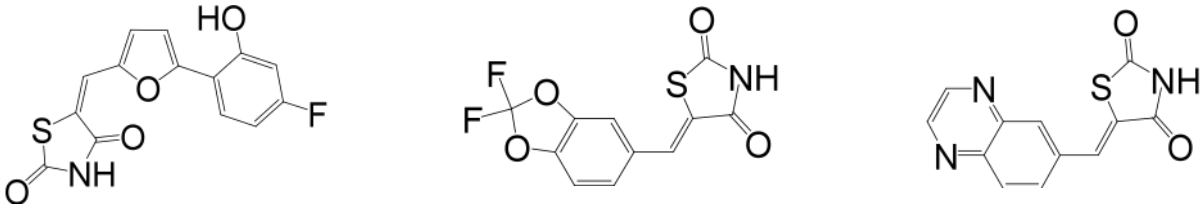


Figure 14: Chemical structure of the PI3Kδ selective inhibitor, IC87114 [566].

Among the second synthetic generation of PI3K inhibitors, AS252424, AS604850 and AS605240 are the first examples of inhibitors selectively targeting PI3Kγ [572] **(Figure 15)**. These inhibitors have been used to block neutrophil chemotaxis *in vitro* and *in vivo* [573]. In mouse models of rheumatoid arthritis, these compounds suppress the progression of joint destruction [574].



AS252424 AS604850 AS605240

Figure 15: Chemical structure of the PI3Kγ selective inhibitors, AS252424, AS604850 and AS605240 [374].

3. Role of PI3K in mast cells

Mast cells express the class IA p85α, p85β and p50α regulatory subunits in addition to all three class IA catalytic subunits p110α, p110β and p110δ and the class IB p110γ associated with the p84/p87 regulatory subunit. Mast cells do not express the regulatory subunit, p101 [395].

3.1. Activation of class I PI3Ks in mast cells

Mast cells express FcεRI, the high affinity receptor for IgE, on their surface, which makes them primary effector cells in allergy, asthma and atopic dermatitis. Binding of antigen to this receptor mediates immediate type I hypersensitivity reaction, leading to the production of inflammatory and vasoactive mediators, lipid-derived mediators, chemokines and cytokines.

Upon multivalent antigen binding to IgE and FcεRI, a signaling cascade is initiated leading to degranulation. Indeed, the ITAMs are phosphorylated by the Src kinase Lyn [575,576] **(Figure 16)**. The tyrosine-phosphorylated ITAMs located in the γ chain-cytoplasmic domains subsequently recruit Syk, via its SH2 domains, which get activated through phosphorylation by Lyn and/or autophosphorylation [577,578]. As a consequence, the transmembrane adapter molecules LAT (linker for activation of T cells) and NTAL (non-T cell activation linker) get phosphorylated and serve as scaffold for multimolecular signaling complexes including adapter molecules Grb2, Gab2 (Grb2-associated binding protein 2) and Gads and the signaling enzymes PLCγ$_1$ and PLCγ$_2$ [579-584]. BMMCs lacking LAT show a severe reduction in Ca^{2+} mobilization, degranulation and cytokine production upon IgE/antigen activation [585]. Once activated, PLCγ catalyzes the hydrolysis of PtdIns(4,5)P_2 that gives rise to DAG and IP_3. DAG associates with the serine/threonine PKC and promotes its activation. IP_3 binds to its receptor on the endoplasmic reticulum and liberate intracellular Ca^{2+} triggering the entry of extracellular Ca^{2+} through stored-operated calcium channels (SOCC) in the plasma membrane [586]. These signals lead to mast cell degranulation, eicosanoid generation and also activation of the transcription factors for chemokine and cytokine production [587].

Degranulation is dependent on the influx of extracellular Ca^{2+}. Indeed, EGTA chelation of extracellular Ca^{2+} abrogates mast cell degranulation [588].

In parallel to this pathway, the cytosolic adapter molecule, Gab2, is phosphorylated by either Fyn or Syk [589,590]. Phosphorylated Gab2 further recruits class IA PI3K via its p85 regulatory subunit through its SH2 and SH3 domains leading to PI3K activation and PtdIns(3,4,5)P_3 production. BMMCs derived from Gab2-deficient mice present defective PI3K-dependent signaling following FcεRI aggregation [591]. Subsequently, molecules containing PH domains, including Vav and Btk (Bruton's tyrosine kinase) can be recruited to the plasma membrane in a PI3K-dependent manner following FcεRI aggregation [592,593].

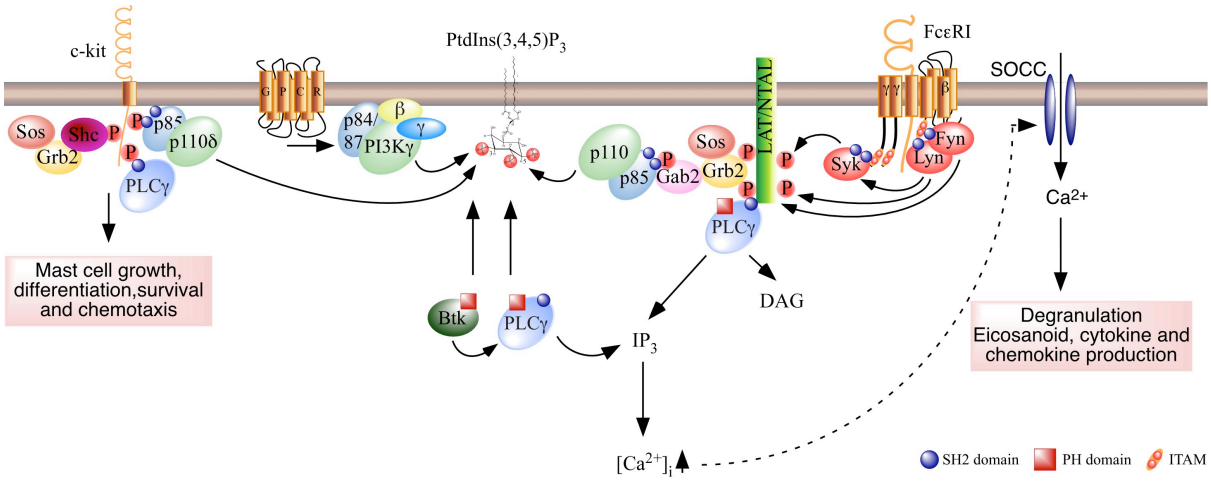


Figure 16: Activation of class I PI3Ks in mast cells. Following FcεRI aggregation, Fyn and Syk become activated resulting in phosphorylation of Gab2, leading to the binding of PI3K class IA by Gab2. A mechanim involving a Btk-dependent phosphorylation of PLCγ results in PKC activation and increase in Ca^{2+} mobilization. LAT and NTAL bind Grb2 following phosphorylation in a Lyn and Syk-dependent manner. Once phosphorylated, LAT and NTAL can also activate PLCγ leading to mast cell degranulation. In the case of c-kit, p85α can directly binds to the phosphorylated receptor and subsequently increases PtdIns(3,4,5)P_3 level via PI3Kδ. Upon ligand binding to GPCRs, PI3Kγ becomes activated and produces PtdIns(3,4,5)P_3 which provides docking site for PH domains of associating proteins [597,598].

PI3K class IA can also be activated via c-kit. Unlike FcεRI, c-kit has inherent protein-tyrosine kinase activity [594]. SCF-mediated dimerization of c-kit induces autophoshorylation at multiple tyrosine residues in the cytoplasmic tail providing docking sites for various molecules including PLCγ_1, Lyn, Fyn, Grb2, Shc and the p85 regulatory subunit. [595]. The

latter leads to activation of PI3Kδ specifically, as the c-kit signaling pathway is impaired in BMMCs derived from p110δ kinase-inactive knock-in mice [570].

Mast cells also express GPCRs such as chemokine and adenosine receptors. Adenosine receptor family is composed of 4 members: A_1, A_{2a}, A_{2b} and A_3 which are all expressed on BMMCs [596]. Adenosine, acting through the A_3 adenosine receptor, increases PtdIns(3,4,5)P_3 level exclusively via PI3Kγ. This stimulation also results in the amplification of IgE/antigen-mediated degranulation of BMMCs [596].

3.2. Pharmacological inhibition of PI3K activity in mast cells

The PI3K inhibitors, wortmannin and LY294002, have been widely reported to inhibit antigen-mediated degranulation in mast cells as well as cytokine production [587,599-601]. Furthermore, wortmannin and the PI3Kγ inhibitor AS252424, also inhibit the antigen-mediated degranulation potentiated by adenosine [571,596]. SCF-mediated signaling in BMMCs can also be impaired either by wortmannin, LY294002 or IC87114, the p110δ selective inhibitor. Indeed, wortmannin and LY294002 inhibit SCF-mediated cell migration, adhesion to FN, proliferation and survival in BMMCs [601]. These attenuated responses are similarly observed with the p110δ-selective inhibitor, IC87114, but not with the p110γ inhibitor, AS252424 [571]. Accordingly, stimulation via FcεRI and c-kit results in a marked PI3K-dependent activation of the mTORC1 pathway as revealed by its wortmannin sensitivity [601].

3.3. Genetic targeting of PI3Ks in mast cells

Disruption of the p85α gene leads to a reduced expression of the class IA PI3K catalytic subunits p110α, p110β and p110δ [602]. The loss of p85α partially inhibited SCF-induced degranulation, proliferation and phosphorylation of PKB. In contrast, p85α gene products were not required for FcεRI-initiated exocytosis and phosphoralation of PKB [602]. Moreover, strong anaphylactic shock with increased vascular permeability and histamine concentration after passive systemic anaphylaxis (PSA) was observed in absence of p85α and was identical to wt animals [603].

Which PI3K isoform is involved downstream of the FcεRI receptor is not clear. The role of the class IB PI3K has been investigated in BMMCs lacking p110γ and indeed, BMMCs derived from PI3Kγ null mice display attenuated degranulation compared to wild-type cells when stimulated through FcεRI receptor. Thus, adenosine-mediated hyperreactivity in BMMCs is abrogated in absence of p110γ. In addition, mice lacking p110γ do not form oedema after intradermal injection of adenosine and show impaired mast cell-mediated allergic response when challenge by PSA [596].

Genetic inactivation of p110δ leads to impaired SCF signaling such as phosphorylation of PKB, adhesion, migration and proliferation [570]. But surprisingly, the loss-of-function of p110δ was also claimed to impaired allergen-IgE-induced degranulation *in vitro* and protects mice against anaphylactic allergic responses [570]. In a recent study, a side-by-side analysis of p110γ and p110δ in mast cell activation was performed using genetic approaches and isoform-selective inhibitors [571]. Inactivation of both isoforms leads to impaired degranulation. As expected, phosphorylation of PKB upon IgE/antigen stimulation is impaired by inactivation of p110γ. Surprisingly, however, inhibition of p110δ is not effective to block the IgE/antigen signaling at first but only at late time point. Even more astonishing, only p110δ inactivation, but not p110γ, shows reduced *in vivo* passive cutaneous anaphylaxis response while *in vitro* both isoform inhibition shows an identical reduction in granule release. These results are quite unexpected as the loss of p85α in mice and mast cells reveals intact FcεRI-mediated signaling *in vitro* and passive systemic anaphylaxis response are identical to the wt animals [602,603].

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AIM OF THE STUDY

The importance of class I PI3Ks activation is known to be essential in mast cell activation as wortmannin, a pan-PI3K inhibitor, blocks histamine secretion and leukotrienes release by irreversibly inhibiting PI3K (Yano et al., 1993). Moreover, the degradation of PtdIns(3,4,5)P_3 by the phosphoinostide phosphatase SHIP, attenuated mast cell activation (Huber et al., 1998). The role of SHIP as a gatekeeper of mast cell degranulation, illustrates that PI3Ks are relevant enzymes in the mast cell-mediated allergic response. The aim of this study was to investigate and to compare the role of PI3Kγ and PI3Kδ in mast cell activation and recruitment *in vitro* and *in vivo*. For this purpose, we performed a step-by-step analysis of mast cell translocation from blood to the inflamed tissue. We used wt, p110γ^{KO} (deficient for p110γ), p110γ^{KR} (p110γ kinase inactivated) and p110δ^{DA} (p110δ kinase inactivated) mice for *in vivo* experiments and BMMCs derived from these mice for *in vitro* experiments as well as the p110δ specific inhibitor, IC87114 and a novel p110γ specific inhibitor, HBC520.

Mast cell recruitment is a process that involves adhesion of mast cell progenitors via α4β1 integrins to endothelial cells via VCAM-1 (Boyce et al., 2002). We were interested to know whether PI3Kγ and PI3Kδ play a role in this first step of the transmigration and we investigated by performing adhesion assay on endothelial cells.

Moreover, mast cells have been described as a source of TNF-α, which is upregulating the cell adhesion molecules VCAM-1 and ICAM-1 on endothelia (Gordon and Galli, 1990; Meng et al., 1995; Plaut et al., 1989). For this reason and the fact that p110δ-deficient mast cells have impaired TNF-α secretion, we speculated that PI3Kγ and PI3Kδ might be involved in VCAM-1/ICAM-1 upregulation as well as in TNF-α and IL-6 production.

Once in tissue, mast cells achieve their differentiation and maturation, and interact with the extracellular matrix. *In vitro* studies have reported that BMMCs can bind to fibronectin but only after activation and induce α5β1 integrin-mediated adhesion to fibronectin. Considering these facts, we performed adhesion assay to test the capacity of PI3Kγ and PI3Kδ to mediate adhesion upon stimulation.

Regarding the IgE/antigen signaling, the role of PI3Kγ and PI3Kδ *in vitro* and *in vivo* is disputed ((Laffargue et al., 2002) vs (Ali et al., 2008)), therefore we investigated their role in FcεRI-driven mast cell degranulation and PKB phosphorylation *in vitro*.

Furthermore, activation through SCF, adenosine, IgE or IgE/antigen mediated BMMCs migration *in vitro* and required functional PI3Kδ and PI3Kγ (Ali et al., 2004; Kitaura et al., 2005). We evaluated the pathophysiologic relevance of these *in vitro* observations by investigating the role of each lipid kinase in mast cell recruitment *in vivo* first by local application of IgE and then testing the IgE/antigen-triggered allergic response.

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RESULTS

Manuscript in preparation

PI3K Modulates Mast Cell Activation in Allergy – from Blood to Tissue

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Running title: Distinct role of PI3Kγ and PI3Kδ in mast cells during inflammation

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1. Abstract

Mast cells are primary effector cells in allergy and chronic inflammation. Class IB (PI3Kγ) and class IA (PI3Kδ) PI3K have been shown to play major roles in mast cell activation: antigen/IgE stimulation triggers autocrine/paracrine activation of mast cells through G protein-coupled receptors (GPCRs) and PI3Kγ, e.g. by adenosine through the A3 adenosine receptor. PI3Kδ acts downstream of c-kit to promote mast cell growth and differentiation. Presently, data concerning the relative importance of PI3Kγ and PI3Kδ are controversial. Here we investigate the role of PI3Kγ and PI3Kδ during mast cell activation and allergic responses. From blood to tissues, we demonstrate that PI3Kγ is a major player in mast cell adhesion to endothelia involving α4β1 integrin whereas adhesion to fibronectin is mediated by α5β1 integrin. VCAM-1 and ICAM-1 upregulation and mast cell-derived TNF-α-mediated activation of endothelia also require functional PI3Kγ. Genetic and pharmacologic approaches confirm the role of PI3Kδ in stem factor signaling (SCF) signaling. However, IgE/antigen signaling and mast cell degranulation are driven by PI3Kγ. Finally, *in vivo* mast cell recruitment as well as passive cutaneous anaphylaxis experiments are severely impaired by absence or pharmacological inhibition of PI3Kγ. Altogether, modulation of PI3Kγ and TNF-α affect mast cell function in crucial phases, rendering them appropriated targets for allergic diseases.

2. Introduction

Mast cells are primary effector cells in allergy and chronic inflammation [1,2]. Derived from circulating hematopoietic progenitor cells, they mature in vascularized tissues [3], and are activated by cross-linking the high affinity IgE receptor (FcεRI) by IgE/antigen complexes [4]. FcεRI is composed of an α-subunit capturing IgE, and a β-subunit and two γ chains mediating downstream signaling [5]. Upon FcεRI cross-linking, the protein tyrosine kinase Lyn phosphorylates immunoreceptor tyrosine-based motifs (ITAMs) located in the cytoplasmic regions of the FcεRI β and γ chains. This initiates the recruitment of Syk and the phosphorylation of multiple tyrosines on LAT, NTAL/LAB [6] and Tyr-X-X-Met motifs on Grb2-associated binder 2 (Gab2; [7,8]). These serve as docking sites for the two src-homology (SH2) domains in class IA phosphoinositide 3-kinase (PI3K) regulatory subunits, which tightly associate with one catalytic subunits of p110α, p110β or p110δ [9-11]. Of these, p110δ was proposed to produce a first wave of the PI3K product PtdIns(3,4,5)P_3 [12].

PI3K activation has been demonstrated to be essential in mast cell activation early on using the pan-PI3K inhibitor wortmannin [13,14], and by the fact that the degradation of PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 by the SH2 domain-containing inositol 5'-phosphtatase (SHIP) attenuates mast cell activation [15]. We have reported previously, that the only class IB PI3K, PI3Kγ, plays a role in mast cell activation in an autocrine/paracrine way: adenosine is released during inflammation and hypoxia and triggers via G-protein coupled receptors (GPCRs) a rapid and transient activation of the PI3Kγ pathway, which potentiates mast cell degranulation initiated by IgE/antigen [16]. Moreover, it has been shown that adenosine activates mast cells in atopic patients [17]. PI3Kγ consists of a catalytic subunit 110γ, which binds to either a p101 [18] or p84 (also called p87^{PIKAP} [19,20] adapter subunit. In mast cells, p84 is predominant and plays the major role in signaling downstream of GPCRs [21]. The output of PI3K and the protein tyrosine kinase cascade finally integrate to trigger the influx of extracellular Ca^{2+}, degranulation of histamine-containing granula, and later, the production of inflammatory mediators [22,23].

Presently, the assigned roles of PI3Kδ and PI3Kγ isoforms in mast cell degranulation are controversial (compare [12,16,24]), and little is known concerning the involvement of PI3Ks in mast cell recruitment. Mast cell recruitment is a highly regulated process, and involves adhesion of mast cell progenitors via α4β1 integrins to endothelial cell adhesion molecules (e.g. VCAM-1, [25]). Mast cells themselves contribute to VCAM-1 and ICAM-1 upregulation by the release of cytokines, including tumor necrosis factor-α (TNF-α, [26-28]). After chemokine-induced extravasation [29], interactions with the extracellular matrix (ECM) promote mast cell differentiation, survival and activation [30,31]. Here, the engagement of α5β1 integrins with fibronectin (FN) have been shown to play a prominent role, and facilitate mast cell activation by IgE/antigen complexes [32-34].
Although it has been shown *in vitro*, that the migration of bone marrow-derived mast cells (BMMCs) is induced by IgE and IgE/antigen complexes involving PI3Kγ signaling [35], the *in vivo* importance of PI3K isoforms in the individual steps leading to mast cell recruitment, degranulation and anaphylaxis, remains still to be defined.

Here, we delineate the involvement of PI3Kδ and PI3Kγ isoforms in the recruitment of mast cells, their cross-talk with endothelia, extravasation and provide *in vitro* and *in vivo* evidence for their selective roles. It remains undisputed that PI3Kδ is essential for signaling downstream of the c-Kit receptor [12], but PI3Kγ takes a prominent role in mast cell adhesion to endothelia, diapedesis and is required for sensitivity of mast cells to IgE/antigen activation in tissues. Functional PI3Kγ also modulates the feedback of tissue mast cells via TNF-α to the activation of endothelial cells. In addition, the combination of genetic and pharmacological approaches revealed that modulation of mast cell recruitment is a promising strategy to attenuate allergic responses. These results have an important impact on the evaluation of currently developed compounds.

3. Materials and Methods

Antibodies and reagents

Phycoerythrin-labeled (PE) hamster anti-mouse FcεRIα (clone MAR-1) antibody was from eBioscience, PE-rat IgG_2b anti-mouse CD117/c-kit (clone 3c1) antibody was from ImmunoKontact. Biotinylated and neutralizing anti-mouse integrin β1 (clone HMb1-1), α4 (clone R1-2), α5 (clone MFR5) antibodies and PE-streptavidin were from Biolegend. PE-mouse $IgG_1\kappa$ anti-human VCAM-1 (clone 51-10C9) antibody was from BD Pharmingen, goat anti-mouse FITC antibody was from Jackson Immuno Research and mouse anti-human ICAM-1 (clone 14D2D12) antibody was a generous gift from Ruggero Pardi. Neutralizing rat IgG_1 anti-mouse TNF-α (clone MP6-XT22) antibody, C5a and SDF-1α were from R&D Systems. Enbrel (etanercept; 25mg in pre-filled syringue), neutralizing rat anti-mouse IgE antibody [36], neutralizing anti-mouse IL-1β (clone 1400.24.17) antibody [37] and HBC520 were from Novartis Institutes for BioMedical research (Basel, Switzerland). CellTracker Green (CTG) and carboxyfluorescein diacetate, succinimidyl ester (CFSE) were from Invitrogen. IC87114 was from Otava Ltd. Evans Blue, Toluidine Blue O, phorbol 12-myristate 13-acetate (PMA), adenosine, N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide, 1-Deoxy-1-[6-[((3-Iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-β-D-ribofuranuronamide (IB-MECA), Dinitrophenyl-human serum albumin (DNP), mouse anti-DNP IgE (clone SPE-7) and cell culture reagents (if not stated differently) were from Sigma-Aldrich. Murine stem cell factor (SCF), murine IL-3 and recombinant murine RANTES were from Peprotech.

Mice

C57BL/6J wild-type (wt) mice were acquired from Jackson, $p110\gamma^{KO}$ [38], $p110\gamma^{KR}$ [39] and $p110\delta^{DA}$ mice [40] in the same genetic background were used, age matched from 8 to 12 weeks. Experiments were carried out in accordance with institutional guidelines and national legislation.

Bone marrow-derived mast cells

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. BM was cultured at 37°C, 5% CO_2 for four days. 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. IL-3 (2ng/ml) was added three times per week [16]. After 4 weeks, BMMC differentiation was monitored by the expression of c-Kit and FcεRI using flow cytometry.

Culture of HUVECs

Human umbilical vein endothelial cells (HUVECs) were cultured in cell culture flasks (BD Falcon) coated with 2% gelatin (type A from porcine skin incubated 30 min at 37°C) in HUVEC medium (M199 medium supplemented with 20% HIFCS, 2 mM L-Gln, 10 μg/ml PEST, 40 U/ml heparin, 20 μg/ml bovine pituitary extract [Gibco]). HUVECs were used between the 4th and 9th passage for experiments.

Adhesion Assays

Preparation of substrats: For BMMC adhesion to endothelia, monolayers of HUVECs were activated with 50 ng/ml TNF-α for 16 hrs at 37°C in 96-well plates coated with 2% gelatin. For BMMC adhesion to fibronectin (FN), 96-well MaxiSorp plates (Nunc) were coated with 1 μg/ml FN in PBS overnight at 4°C, blocked on the following day with 4% BSA in PBS for 1 hr at 37°C, followed by a rinse with PBS.

Preparation of cells: For DNP stimulations, BMMCs were preloaded overnight with 0.5 μg/ml IgE at 37°C. For other agonists, non-sensitized BMMCs were used. BMMCs in PBS were labeled with 10 μM CFSE for 10 min at 37°C. The cells were then washed twice in IMDM/BSA (IMDM/L-Gln/PEST/50 μM β-mercaptoethanol plus 1% BSA and 20 mM Hepes, pH 7.4). Where indicated, BMMCs were then pre-incubated with neutralizing antibodies for 30 min at 37°C. The stimulants were added to 96-well plates in 50 μl IMDM/BSA followed by the addition of labeled and pretreated $5x10^4$ BMMCs in 100 μl IMDM/BSA. After 30 min of incubation at 37°C, each well was washed three times with modified Tyrodes buffer (137 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/5.6 mM glucose/0.1 % BSA/20 mM Hepes, pH 7.4) under constant flow conditions (electronic multichannel pipetman, speed 1 [Gilson]) to remove non-adherent cells. Finally, the

fluorescence of adherent cells was determined (excitation: 492nm; emission: 518nm) and expressed as percentage of the fluorescence intensity of initially added cells.

FACS Analysis

Adhesion molecules on HUVECs: Confluent HUVECs were incubated with 5 times diluted BMMC supernatant (Sn) at 37°C for 16 hrs (time corresponding to the peak expression of VCAM-1/ICAM-1) [28]. Where mentioned, BMMC supernatant was pre-incubated with PBS, Enbrel, neutralizing anti-TNF-α or anti-IL-1β antibodies for 1 hr at 37°C and then added to HUVECs. Stimulated HUVECs were trypsinized and incubated with PE-labeled anti-VCAM-1 or anti-ICAM-1 antibodies for 30 min at 4°C. For ICAM-1 expression, the cells were subsequently incubated with secondary FITC-labeled anti-mouse antibodies for 30 min at 4°C.

Integrins on BMMCs: BMMCs were incubated with biotinylated anti-β1, anti-α4 or anti-α5 integrin antibodies for 30 min at 4°C, and then incubated with PE-streptavidin for 20 min at 4°C. All flow cytometry acquisitions were performed with FACS Calibur (BD) and the data were analyzed with Flowjo (Treestar).

BMMC stimulation and cytokine secretion

BMMCs were preloaded overnight with 100 ng/ml IgE, washed, re-suspended at $1x10^6$ cells/ml, and stimulated with 5 ng/ml DNP or 5 ng/ml DNP plus 1 μM adenosine for 6 hrs at 37°C. Where indicated, wt BMMCs were incubated for 1 hr with inhibitors or 0.1% DMSO before stimulations. The cells were harvested, and the amount of cytokines in BMMC supernatants was measured by specific ELISA kits from R&D Systems according to the manufacturer protocol.

Cell migration assay

Migration of BMMCs was assayed for 6 hrs in 24-well Transwell Supports (Corning; 5.0 μm pore polycarbonate membranes bottom coated overnight with 1 μg/ml FN in PBS at 4°C, blocked with 4% BSA in PBS for 1 hr at 37°C, and equilibrated in IMDM/BSA for 30 min at 37°C). Transwells containing $0.25x10^6$ BMMCs in 200 μl IMDM/BSA were immersed in the lower well containing stimulants in 500 μl IMDM/BSA. Cells migrated to the lower well were counted using a Neubauer Chamber.

***In vivo* mast cell recruitment:**

Toluidine Blue O staining: Mice were shaved on the back one day before injections. Subsequently, isotype control or neutralizing compounds (anti-IgE, anti-IL-1β antibodies or Enbrel) were injected intraperitoneally (i.p.), and 12 hrs later IgE or PBS were injected intradermally (i.d.) into the flank. Mice were injected i.d. with 5 μl IgE (100 ng) or PBS into the ear or 20 μl IgE (100 ng) or PBS in the left and right flank respectively. Skin biopsies from IgE injection and control sites were obtained 24 and 48 hrs later. The tissues were fixed, paraffin-embedded and stained with 0.5% Toluidine Blue O solution to assess the presence of tissue mast cells (MCs). MCs were counted per injection site (6 slides per specimen from 3 different animals per each condition) using a light microscope with a 12.5 x 12.5 μm grid at x20 magnification and average MCs/mm^2 was determined by blinded counting.

Intravital microscopy: C57BL/6 mice received an intrascrotal injection of TNF-α (50 ng diluted in 400 μl PBS). $5x10^6$ CTG-labeled BMMCs were applied via cannulated left femoral artery 2 hrs later. Surgical preparation of the right cremaster muscle as well as *in vivo* fluorescence microscopy was performed 2 hrs later as described earlier [41,42]. Rolling and firmly adherent BMMCs were counted in five fields of view covering ~ 70 – 80 % of the entire cremasteric surface and given as average number of cells per minute and $10mm^2$ surface area. Rolling mast cells were defined as those moving slower than the associated blood flow. Firmly adherent cells were determined as those resting in the associated blood flow for more than 30 seconds.

Degranulation Assay:

BMMCs were preloaded overnight with 100 ng/ml IgE, washed twice and re-suspended at $1x10^6$ cells/ml in modified Tyrodes buffer. Degranulation was induced by the indicated DNP concentrations, and the reaction was stopped after 20 min by addition of 0.1 M $Na_2CO_3/NaHCO_3$. Where indicated, wt BMMCs were incubated for 30 minutes with inhibitors or 0.1% DMSO before DNP stimulation. β-hexosaminidase activity in supernatants was assessed with *p*-nitrophenyl N-acetyl-β-D-glucosaminide (*p*-NAG) as a substrate at 410 nm. Results are expressed as the percentage of total TritonX-100 releasable β-hexosaminidase in whole cells [16].

Immunoblotting:

Proteins were separated by SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore). Rabbit monoclonal anti-pSer473-PKB/Akt (Cell Signaling Technology), mouse monoclonal anti-PKB/Akt (clone 19G7/C7), monoclonal anti-p110γ (Alexis; clone H1) and rabbit polyclonal anti-p110δ (Santa Cruz; clone H-219) were used to detect molecules in the PI3K pathway. Secondary antibodies such as horseradish peroxidase (HRPO)-coupled rabbit anti-mouse IgG and goat anti-rabbit IgG antibodies (Sigma-Aldrich) were visualized using enhanced chemiluminescence (Millipore).

Passive Cutaneous Anaphylaxis (PCA):

Mice were shaved on the back one day before injections. They then received i.d. injections of 20 μl of PBS and IgE in the top and bottom back skin respectively. An i.v. injection of DNP in 100 μl of 0.5% Evans Blue in saline solution was performed 48 hrs later, and skin biopsies from IgE injection and control sites were obtained 30 min later. Evans blue was extracted in 300 μl formamide at 55°C for 48 hrs, and quantified at 620 nm. Where indicated, inhibitors or vehicle (0.5% methylcellulose/Tween 80) were administrated per os (p.o.).

4. Results

Mast cells are localized throughout all tissues, and are abundant in skin and mucus membranes. Mast cell numbers can be dynamically regulated, and allergic and inflammatory conditions, as well as host defense mechanisms can provoke the recruitment of mast cells. Asthma, allergic rhinitis, atopic dermatitis are often accompanied by elevated levels of IgE [43], and it has been reported that IgE can trigger mast cell migration *in vitro* [35].

IgE-induced mast cell recruitment to skin requires p110γ but not p110δ.

Here, we determined the number of mast cells at specific dermal sites in wild type (wt), $p110\gamma^{-/-}$ (abbreviated as $p110\gamma^{KO}$ in the following) and $p110\gamma^{K833R/K833R}$ (short $p110\gamma^{KR}$) and $p110\delta^{D910A/D910A}$ ($p110\delta^{DA}$) mice before and after a challenge with IgE. While unchallenged mice of all genotypes displayed indistinguishable mast cell numbers in dorsal skin, animals with inactivated $p110\delta^{DA}$ had only half the mast cells in the ear (Fig. 1A). These results are in agreement with previously published data in $p110\gamma^{KO}$ [16] and $p110\delta^{DA}$ mice [24].

When IgE was injected intradermally (*i.d.*), this recruited mast cells to the injection site, while PBS injections were ineffective (Fig. 1B, C). The examination of Toluidine-stained histological samples illustrated a significant increase in mast cell numbers at 24 h and close to a doubling of resident mast cells at 48 h in ears and dorsal skin of wild type mice. Interestingly, this IgE-induced mast cell infiltration was completely abrogated in the absence of functional p110γ ($p110\gamma^{KO}$ and $p110\gamma^{KR}$), but very little affected in $p110\delta^{DA}$ mice. These results suggest that p110γ but not p110δ is required for the acute, IgE-induced recruitment of mast cells to tissues, which is in agreement with the requirement of p110γ in *in vitro*-mediated mast cell migration triggered by IgE [35]. Starting from lower numbers of resident mast cells in the ears of $p110\delta^{DA}$ animals, IgE triggered the same proportional increase as compared to wild type (1.8 x), but did not give rise to maximal mast cell numbers as detected in challenged wild type ears. As IgE targets specifically mast cells, this might be the first indication that resident tissue mast cell numbers are relevant for the further progression of IgE-mediated responses.

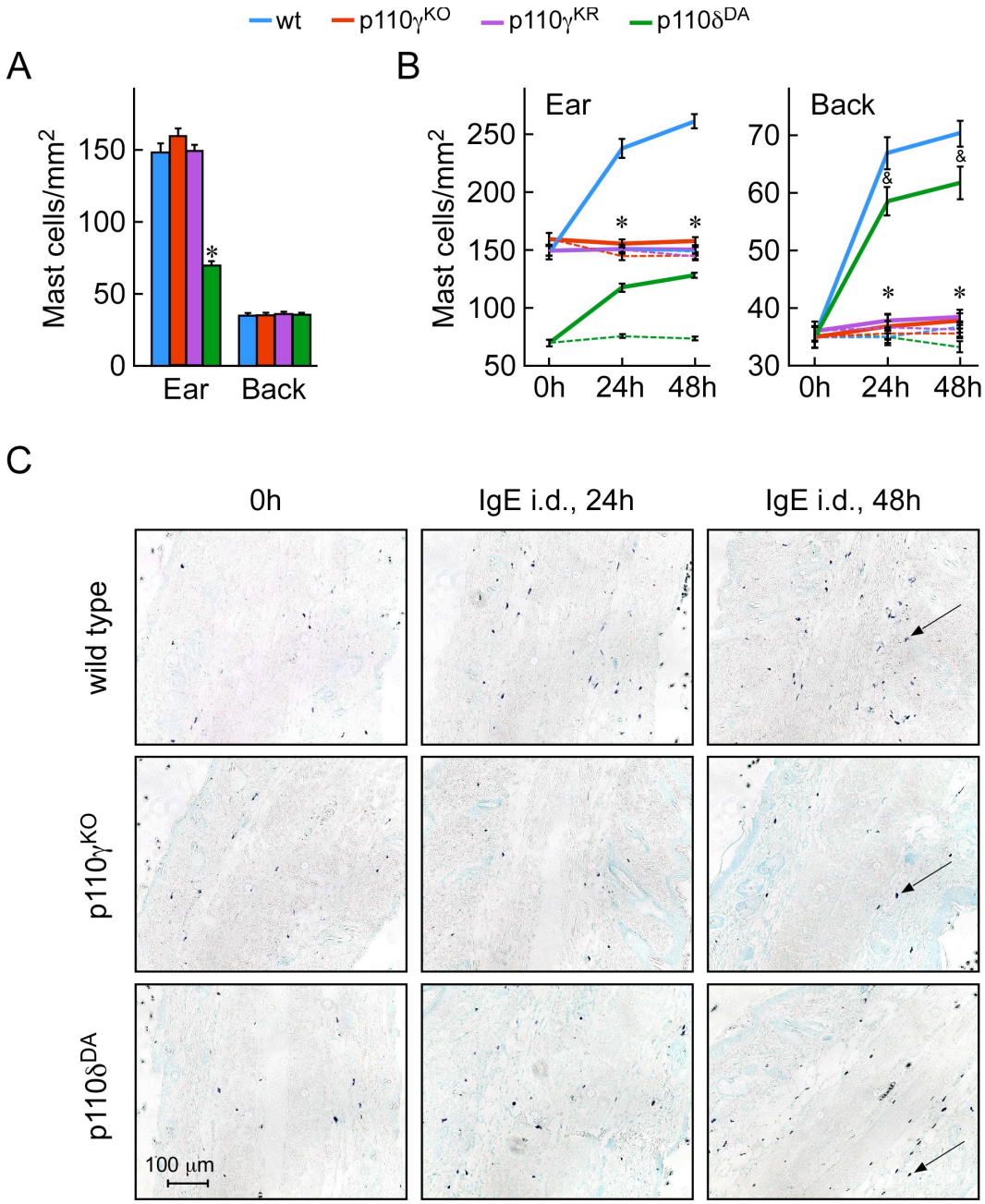

Figure 1. IgE-induced mast cell recruitment is p110γ-dependent *in vivo*. (A) Ear and back skin biopsies from wt, $p110\gamma^{KO}$, $p110\gamma^{KR}$ and $p110\delta^{DA}$ mice were prepared, stained with Toluidine Blue O and the mast cells (MCs) number/mm^2 were determined by light microscopy (n>3, mean ± SEM, * p<0.001 compared with wt). (B) Wt, $p110\gamma^{KO}$, $p110\gamma^{KR}$ and $p110\delta^{DA}$ mice were i.d. injected with 100 ng IgE (plain lines) or PBS (dotted lines) and killed 24 or 48 hrs later. MCs were counted by light microscopy. (n>3, mean ± SEM, & p<0.05, * p<0.001 compared with wt). (C) Representative pictures of wt, $p110\gamma^{KO}$ and $p110\delta^{DA}$ back skin biopsies. The arrows show MCs stained with Toluidine Blue O.

Agonist-dependent roles for p110γ and p110δ in BMMC migration.

To analyze the relay of signals through p110γ and p110δ during mast cell recruitment, *in vitro* migration assay were performed (Fig. 2A). In transwell assays, mast cell migration to lower wells containing SCF, adenosine, the A3 adenosine receptor (A3AR) agonist IB-MECA or IgE with and without antigen was assessed after 6 h of exposure to stimuli. As expected, BMMCs derived from $p110\delta^{DA}$ mice completely lost their capability to migrate towards SCF, while the absence of p110γ did not affect SCF signaling trough c-Kit. The situation was reversed with the GPCR ligands adenosine and IB-MECA, which required functional p110γ for migration, while p110δ function was dispensable here.

As reported by Kitaura [35], IgE or IgE/antigen complexes can trigger mast cell migration *in vitro* involving autocrine/paracrine signaling through p110γ. The selectivity for the requirement of the p110γ isoform could be confirmed here, as both, the migration towards IgE and IgE/antigen were abrogated in $p110\gamma^{KO}$ BMMCs, while the inactivation of p110δ did not significantly affect the migratory process *in vitro*.

Consequently, it appears that the IgE or IgE/antigen triggered activation of mast cells mainly causes the release and action of adenosine and other GPCR ligands [16,35], and that cytokines signaling through p110δ play a minor role. Combined with the intact *in vivo* recruitment of $p110\delta^{DA}$ mast cells, one may conclude that SCF signaling plays no role in the IgE-induced mast cell infiltration to dorsal sites.

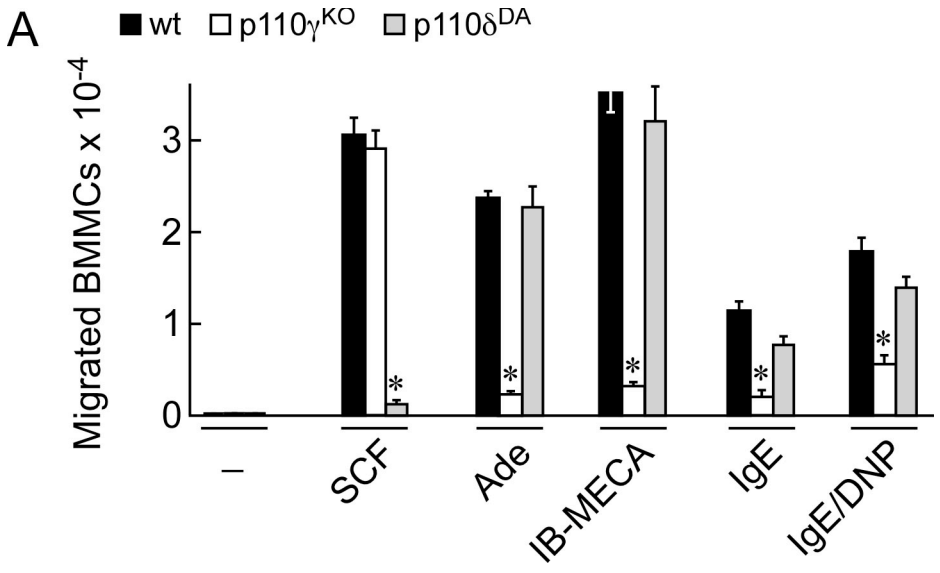

Figure 2. Differential roles for p110g and p110d regarding BMMC migration. (A) BMMCs from wt, p110γ^{KO} and p110δ^{DA} mice were stimulated with 20 ng/ml SCF, 1 μM adenosine, 10 nM IB-MECA, 1 μg/ml IgE or 100 ng/ml IgE overnight plus 10 ng/ml DNP and allowed to migrate through transwell-coated with FN for 6 hrs at 37°C. Migrated cells in the lower well were counted (n>3, mean ± SEM, * p<0.001 compared with wt).

BMMC adhesion to endothelial cells is mediated by PI3K.

Adhesion of immune cells to endothelial cells is a prerequisite for immune cell migration to target tissues. To explore the involvement of PI3Ks in this process, we used stimuli known to attract leukocytes to inflammatory sites. C5a, SDF-1α, RANTES, as well as adenosine and IB-MECA were tested for their capability to induce BMMC adhesion to HUVECs *in vitro* (Fig. 2B). These GPCR agonists all induced the adhesion of wild type and p110δ^{DA} BMMCs to HUVECs, while p110γ null BMMCs did not respond. SCF could also be used to trigger adhesion of BMMCs to HUVECs, and here all mast cells except for the p110δ^{DA} BMMCs did adhere.

Next we assessed, which adhesion molecules mediated the interaction between BMMCs and endothelia. Boyle *et al.* [25] have demonstrated that the adhesion of human progenitor mast cells to HUVECs can be blocked using anti-α4-integrin antibodies. As shown in Figure 2C, resting BMMCs from wild type, p110γ^{KO}, and p110δ^{DA} mice expressed α4, α5 and β1 integrins on their surface at identical levels.

When BMMCs were exposed to anti-integrin blocking antibodies before stimulation, anti-α4, anti−β1 and combinations of anti-α4 and anti−β1 antibodies were most efficient to prevent mast cell adhesion to endothelia, while anti-α5 antibodies had negligible effects, and anti-α5/anti−β1 combinations were not superior to anti−β1 antibodies alone (Fig. 2D). As blockage of adhesion was mediated for p110γ and p110δ signaling by targeting α4β1 (VLA-4) integrins, this illustrates that the signaling of both PI3K isoforms can converge into the same cell responses, but that the two pathways are clearly separated upstream of PtdIns(3,4,5)P_3 production. And although it occurs *in vitro,* the p110δ-dependent increase in adhesion does not seem to play a role *in vivo*.

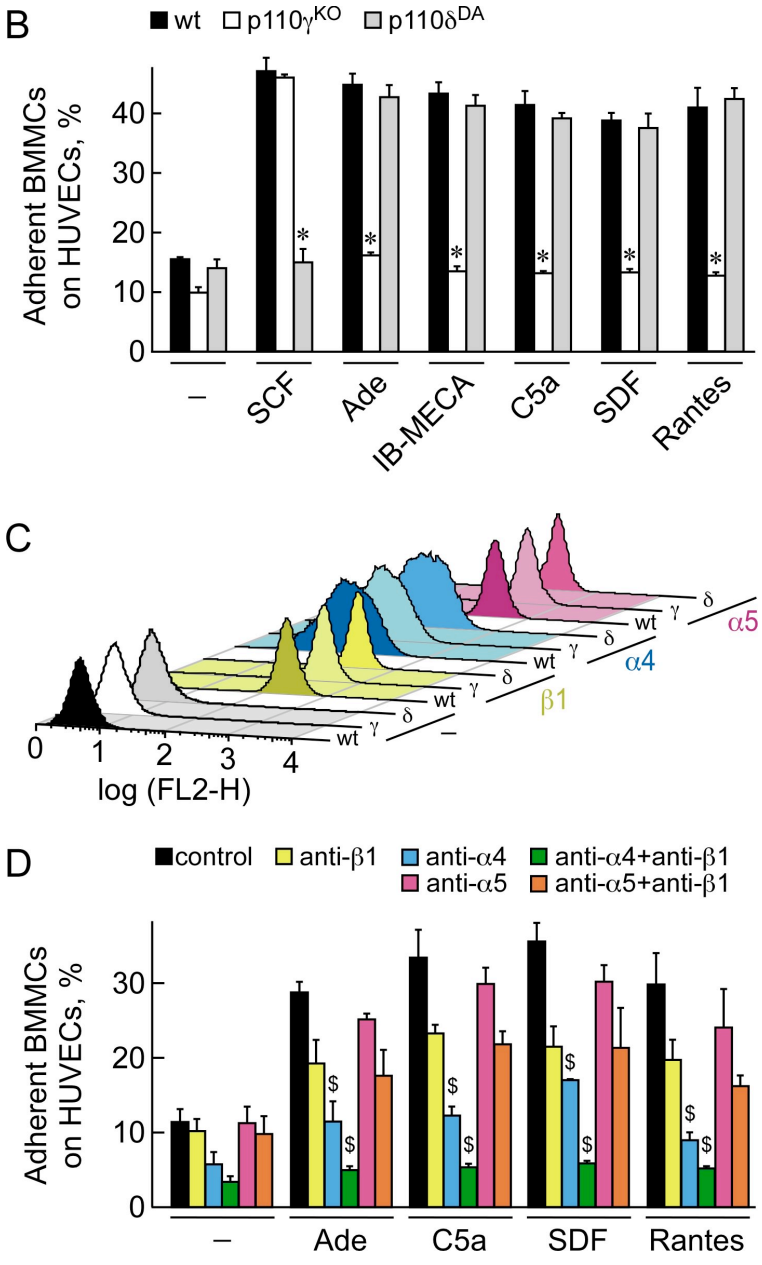

Figure 2. Chemokines-induced adhesion of BMMCs to HUVECs is p110γ-dependent. (B) CFSE-labeled BMMCs from wt, p110γ^{KO} and p110δ^{DA} mice were incubated on HUVECs with 20 ng/ml SCF, 1 μM adenosine, 10 nM IB-MECA, 10 nM C5a, 10 nM SDF-1α or 10 nM RANTES for 30 min at 37°C, washed and fluorescence of adherent cells was measured (n>3, mean ± SEM, * p<0.001 compared with wt). (C) Integrin expression in BMMCs: cells were incubated with anti-α4, anti-α5 and anti-β1 biotinylated antibodies for 30 min at 4°C, then with streptavidin-PE for 20 min at 4°C and analyzed by FACS. (B) Wt BMMCs were incubated with neutralizing anti-α4, anti-α5 and anti-β1 integrin antibodies (40 μg/ml) for 30 min at 37°C before being seeded on HUVECs and adhesion assay was performed as mentioned above (n>3, mean ± SEM, $ p<0.01 compared with control).

Differential roles for p110γ and p110δ mediating adhesion of BMMCs to extracellular matrix protein fibronectin.

After the transit to tissues, mast cells responsiveness is regulated by interactions with extracellular matrix proteins such as fibronectin (FN). To address the role of PI3Ks in this event, we assessed adhesion of wild type, $p110\gamma^{KO}$ and $p110\delta^{DA}$ BMMCs to FN-coated plates. Resting BMMCs do not adhere to FN, but BMMCs of all origins adhered after exposure to the protein kinase C activator phorbol 12-myristate 13-acetate (PMA; Fig. 3A). As for the adherence to endothelial cells, activation of c-Kit via SCF required a functional p110δ to achieve adhesion, which confirms previously published data [24], while for GPCR ligands (adenosine, IB-MECA) p110γ was essential to fix mast cells on FN. Exposure of BMMCs to IgE alone was sufficient to trigger adhesion to FN. This response was completely abrogated in p110γ-deficient cells, while loss of p110δ function reduced adhesion insignificantly as compared to wild type cells. When the input signal to FcεRI was increased using IgE/antigen complexes, elimination of p110γ was less efficient to obstruct adhesion (Fig. 3A).

Adhesion of activated BMMC to FN is known to be mediated by a high-affinity state of α5β1 integrins [33,34,44,45], but little is known about which PI3K isoforms couple to increased ligand binding of α5β1 integrins. As anti-α5 integrin and the combination of anti-α5 and anti-β1 integrin antibodies blocked PMA, SCF and adenosine triggered adhesion to FN most efficiently, both p110γ and p110δ can basically provide the signals to activate α5β1 integrins, but signals emerge from different input and are relayed by clearly separated pathways (Fig.3B). Along these lines, it was also demonstrated, that SCF-induced mast cell adhesion to FN was insensitive to inhibitors selectively targeting p110γ (AS252424; [24]).

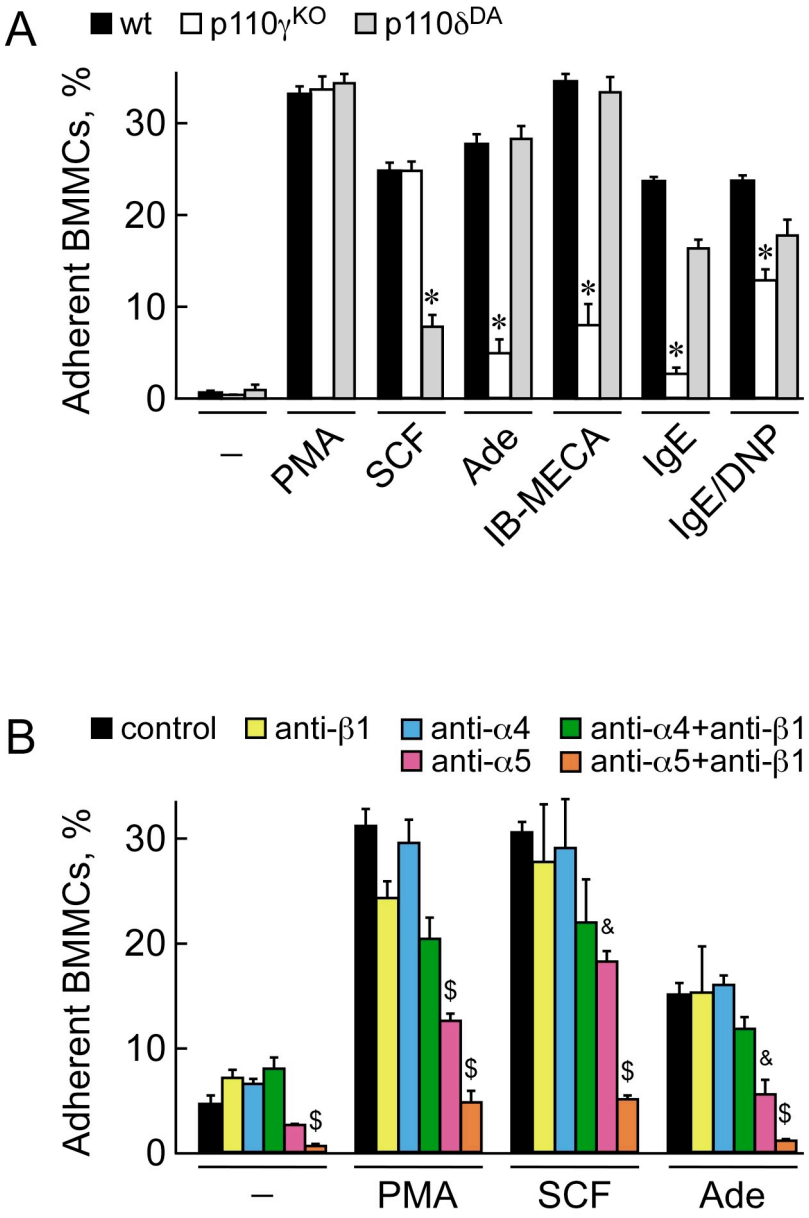

Figure 3. Differential roles for p110γ and p110δ regarding BMMC adhesion on fibronectin. (A) CFSE-labeled bone marrow-derived mast cells (BMMCs) from wt, $p110\gamma^{KO}$ and $p110\delta^{DA}$ mice were stimulated with 100 nM PMA, 20 ng/ml SCF, 1 μM adenosine, 10 nM IB-MECA, 1 μg/ml IgE or 500 ng/ml IgE overnight plus 100 ng/ml DNP. After 30 min incubation on FN at 37°C, wells are washed, and fluorescence of adherent cells was measured (n>3, mean ± SEM, * $p<0.001$ compared with wt). (B) Wt BMMCs were pre-incubated with neutralizing anti-α4, anti-α5 and anti-β1 integrin antibodies (40 μg/ml) for 30 min before being seeded into FN-coated plates. Fluorescence of the adherent cells was measured (n=3, mean ± SEM, & $p<0.05$, $ $p<0.01$, * $p<0.001$ compared with control).

Mast cells require p110γ to activate and bind to endothelial cells.

Meng et al. [28] have shown previously that supernatants from activated BMMCs (BMMC Sn) markedly increase surface expression of the adhesion molecules ICAM-1 and VCAM-1 on endothelial cells. To evaluate the need for PI3K signaling, supernatants from wild type, $p110\gamma^{KO}$ and $p110\delta^{DA}$ BMMCs stimulated with IgE/ antigen or IgE/antigen and adenosine, were transferred to HUVECs. As detected by FACS, the exposure of VCAM-1 and ICAM-1 after 16 hours incubation with BMMC Sn was maximal in wild type cells (Fig. 4A, B), and could be mimicked by the addition of excess TNF-α to HUVECs [46,47]. VCAM-1 upregulation was efficiently induced by supernatants from stimulated wild type and $p110\delta^{DA}$ BMMCs, while the loss of p110γ reduced the potency of BMMCs supernatants close to background activity. ICAM-1 levels were already elevated in non-stimulated HUVECs, and BMMC supernatants roughly doubled ICAM-1 surface expression, except for $p110\gamma^{KO}$ BMMC-derived supernatants with a small, but significant reduction in activity.

When supernatants from stimulated BMMCs were supplemented with anti-TNF-α antibodies or the TNF-α blocker Enbrel (a soluble fusion protein combining two type II TNF-α receptor [TNFR2] with an Fc fragment of human IgG1 [48]) before they were added to HUVECs, VCAM-1 and ICAM-1 upregulation on endothelial cells was completely abrogated. The addition of IL-1β neutralizing antibodies prior to incubation with HUVECs did not interfere with VCAM-1/ICAM-1 upregulation (Fig. 4C and D). This indicates, that the upregulation of VCAM-1 and ICAM-1 on endothelial cells is mediated mainly by TNF-α and that mast cells require mainly p110γ to produce this cytokine in response to IgE stimulation. To verify this, secretion of TNF-α and IL-6 from BMMCs stimulated with combinations of IgE, antigen and adenosine was determined. The loss of p110γ or its inhibition with the p110γ-selective inhibitor HBC520 in wild type BMMCs (see fig. S2 for the characterization of the inhibitor) ablated TNF-α production, and reduced IL-6 secretion by >50-60% (Fig. 5A and B). Interference with p110δ activity by genetic ($p110\delta^{DA}$) and pharmacological means (IC87114, [49]) reduced TNF-α and IL-6 release somewhat after stimulation with IgE/antigen, but was ineffective when adenosine was combined with IgE/antigen stimulation. As the latter condition best fits the *in vivo* situation during an allergic response where adenosine is released, one might expect that p110γ plays a dominant role in the release of the two above mentioned cytokines.

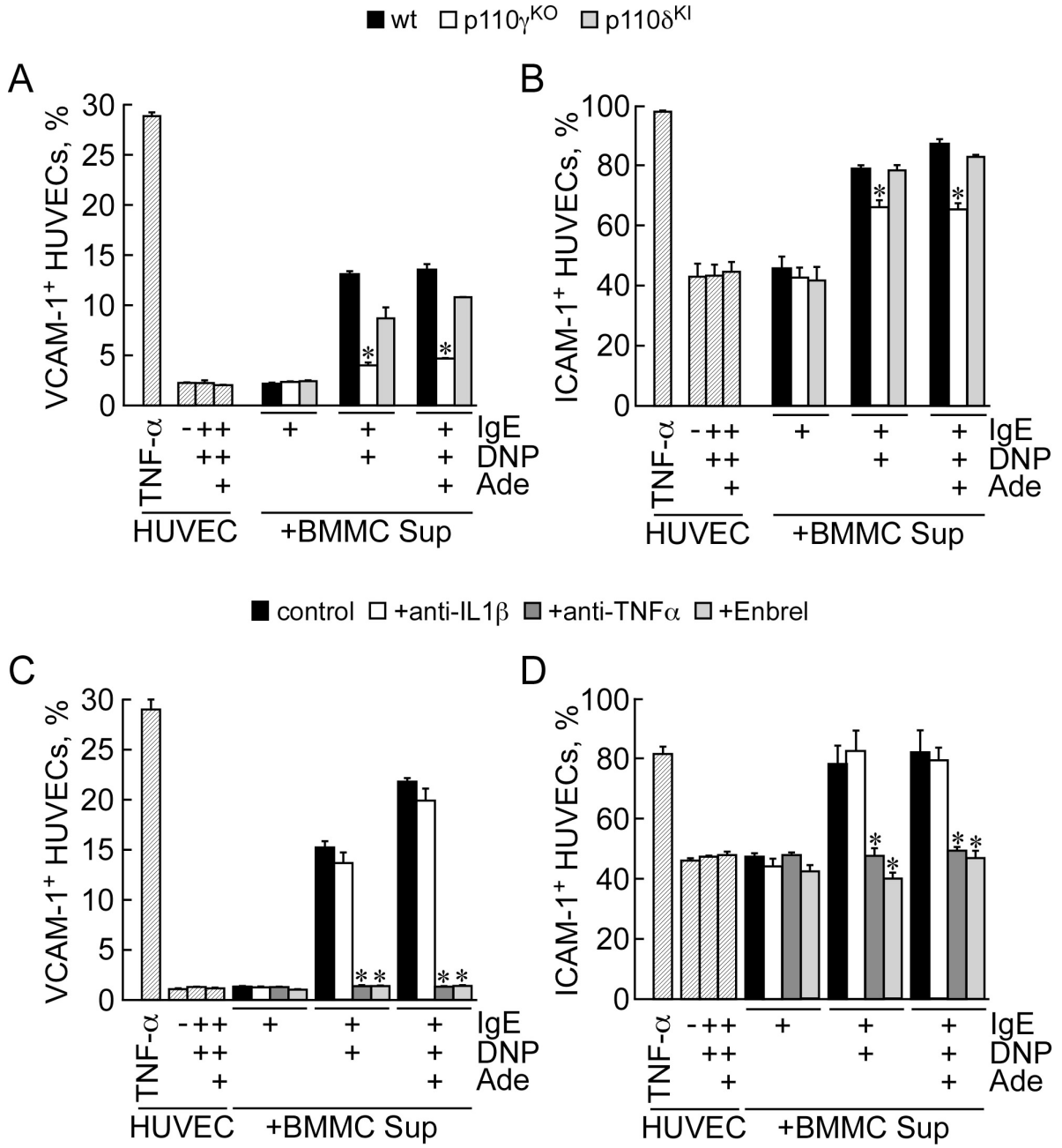

Figure 4. VCAM-1/ICAM-1 expression on endothelia is mediated by TNF-α and is dependent on the presence of p110γ in BMMCs.

Upregulation of VCAM-1 (A) and ICAM-1 (B) on HUVECs is induced after 16 hrs exposure to BMMC supernatant (Sn). Cells were incubated with anti-VCAM-1 and anti-ICAM-1 antibodies and then analyzed by flow cytometry (n>3, mean ± SEM, * $p<0.001$ compared with wt). HUVECs were exposed to BMMC Sn previously incubated with 1μg/ml anti-TNF-α antibody, 1μg/ml of anti-IL-1β antibody or 10μg/ml Enbrel for 1 hr at 37°C. VCAM-1 (C) and ICAM-1 (D) surface expression were analyzed by flow cytometry (n=3, mean ± SEM, * $p<0.001$ compared with control).

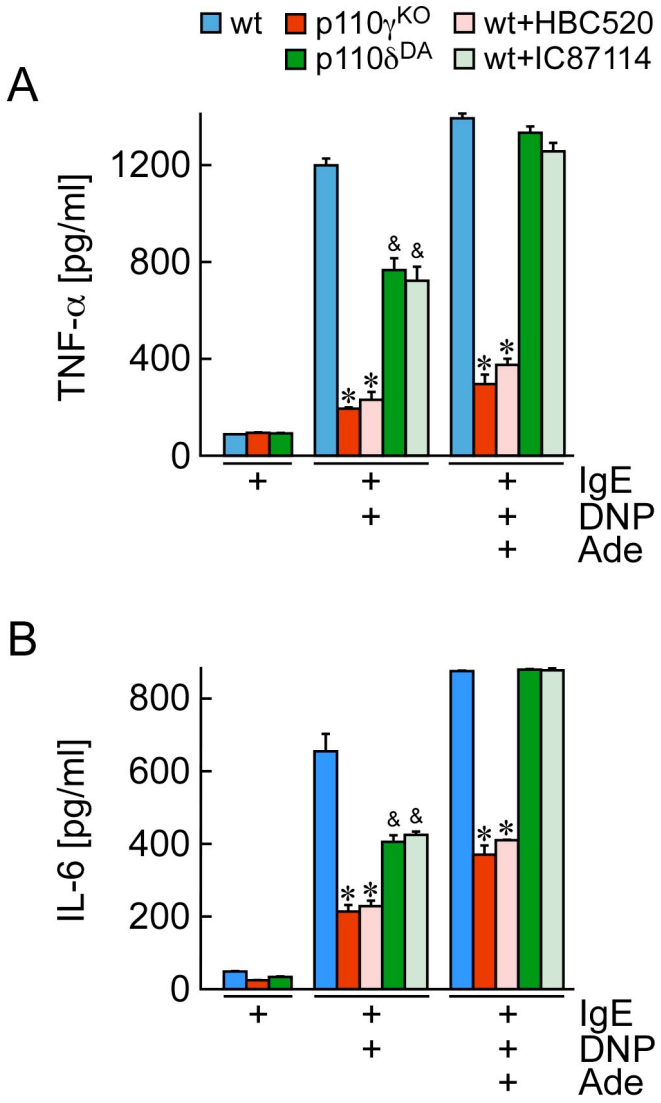

Figure 5. Optimal TNF-α and IL-6 production require p110γ in BMMCs. (A) TNF-α and (B) IL-6 release from BMMCs after overnight incubation with 100 ng/ml IgE and stimulation with 5ng/ml DNP or with 5ng/ml DNP plus 1 μM adenosine for 6 hrs. HBC520 and IC87114 were used at 5μM and added for 1hr before stimulations (n=3, mean ± SEM, * $p<0.001$, & $p<0.05$ compared with wt).

IgE-mediated PKB/Akt phosphorylation and degranulation require p110γ.

It has been demonstrated that p110δ is the major source of PI3K activity downstream of the c-Kit receptor in SCF stimulated BMMCs [24]. This and the independence of SCF-stimulated phosphorylation of PKB/Akt on p110γ was confirmed here (Fig. 6A).

BMMCs stimulated by IgE/antigen exhibit a phosphorylation of PKB/Akt that is abrogated to a big extent in $p110\gamma^{KO}$ BMMCs, while inactive p110δ does not show a significant decrease in PKB/Akt phosphorylation (Fig. 6B), a differences maintained over time. The PKB/Akt phosphorylation is mirrored by the relative contributions of p110γ and p110δ in the IgE/antigen-mediated degranulation response (as measured by the release of β-hexosaminidase), where degranulation was impaired in $p110\gamma^{KO}$ BMMCs [16], and was likewise prevented by two p110γ specific inhibitors (HBC520, and AS252424 [50,51], see Fig. 6C). The inactivation of p110γ attenuates degranulation most efficiently at low antigen concentrations, while high antigen concentrations were capable to override the block imposed by p110γ inhibition partially. In contrary to its role downstream of c-kit, we could not establish a role for p110δ in FcεRI-induced signaling (see also fig. S2), which stands in opposition to results obtained by Ali et al. [12].

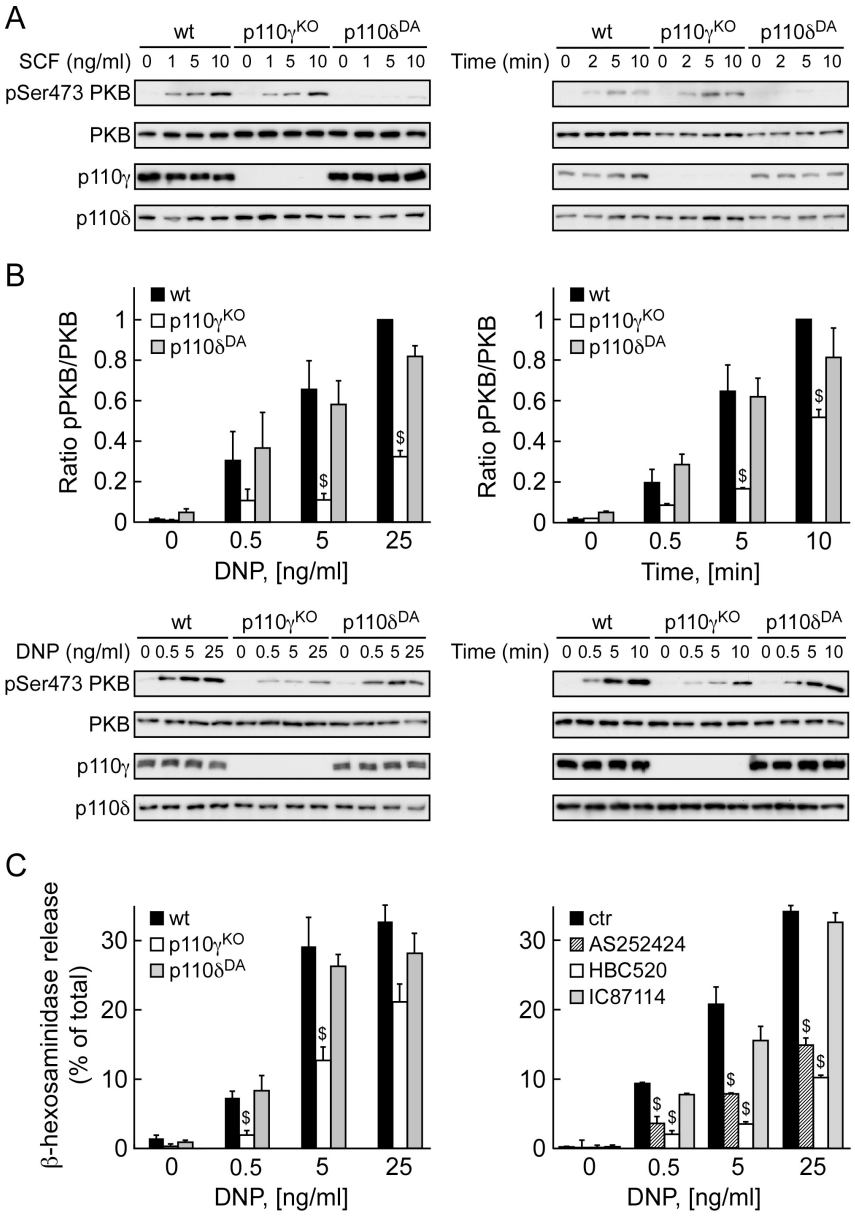

Figure 6. Contribution of p110γ and p110δ to SCF and FcεRI-induced BMMC responses. (A) BMMCs from wt, p110γ^{KO} and p110δ^{DA} mice were starved in medium containing 2% FCS for 3 hrs and stimulated with different SCF concentrations (left panel) and for different times (right panel). (B) BMMCs from wt, p110γ^{KO} and p110δ^{DA} mice were incubated overnight with 100 ng/ml IgE, starved and stimulated with different DNP concentrations (left panel) and for different times (right panel). The ratio pPKB/PKB have been quantified using ImageJ (n=3, mean ± SEM, $ $p<0.01$ compared with wt). (C) Degranulation was followed by β-hexosaminidase activity in wt, p110γ^{KO} and p110δ^{DA} BMMCs (left panel) or in wt BMMCs pre-incubated with inhibitors (right panel) after overnight incubation with 100 ng/ml IgE. Cells were stimulated for 20 min with the indicated amount of DNP before β-hexosaminidase was assessed in the cell supernatant. HBC520 and AS252424 were used at 5μM and IC87114 at 10μM and added for 30 min before DNP stimulation (n>3, mean ± SEM, $ $p<0.01$ compared with wt or control).

Chronic inhibition of p110γ best to suppress passive anaphylactic responses.

To test the validity of the *in vitro* results above, wild type, $p110\gamma^{KO}$, $p110\gamma^{KR}$ and $p110\delta^{DA}$ mice were subjected to a model of passive cutaneous anaphylaxis (PCA). For PCA, mice were locally sensitized with *i.d* injections of IgE or PBS in the dorsal dermis, and an *i.v.* application of antigen (DNP) and Evans blue followed 48 hours later. The extravasation of Evans Blue was then measured to quantify the allergic response manifested as mast cell-induced vascular permeability. When the doses of IgE or antigen were stepwise increased, the PCA response was augmented in wild type mice (Fig. 7A). At elevated IgE concentrations, inactivation of p110δ rapidly failed to protect mice against PCA, while $p110\gamma^{KO}$ mice still showed robustly attenuated responses. When antigen levels were gradually increased, it became apparent, that also the block imposed in $p110\gamma^{KO}$ and $p110\gamma^{KR}$ mice could be partially bypassed, although a 40-50% protection remained even at elevated antigen concentrations (Fig. 7B).

Of two orally available, isoform-specific PI3K inhibitors, it was only HBC520 targeting p110γ, which blocked PCA responses efficiently. The inhibition of p110δ by IC87114 was ineffective in our hands, although the compound was previously claimed to attenuate anaphylaxis [12]. During our studies it became also clear, that a chronic treatment (for the injection scheme see Fig. 7C) with HBC520 was superior to even 10 times elevated doses of the inhibitor applied acutely (Fig. 7D). Interestingly, also Enbrel applied over the sensitization period was able to prevent a PCA response. As shown in Fig. 7E, the efficiency of the interference with PCA appeared to correlate with the recruitment of mast cells to challenged tissue. Mimicking the situation in $p110\gamma^{KO}$ and $p110\gamma^{KR}$ mice, the chronic treatment with HBC520 prohibited IgE-induced mast cell accumulation, and chronic IC87114 had a moderate but significant effect as observed for mast cell recruitment in $p110\delta^{DA}$ animals.

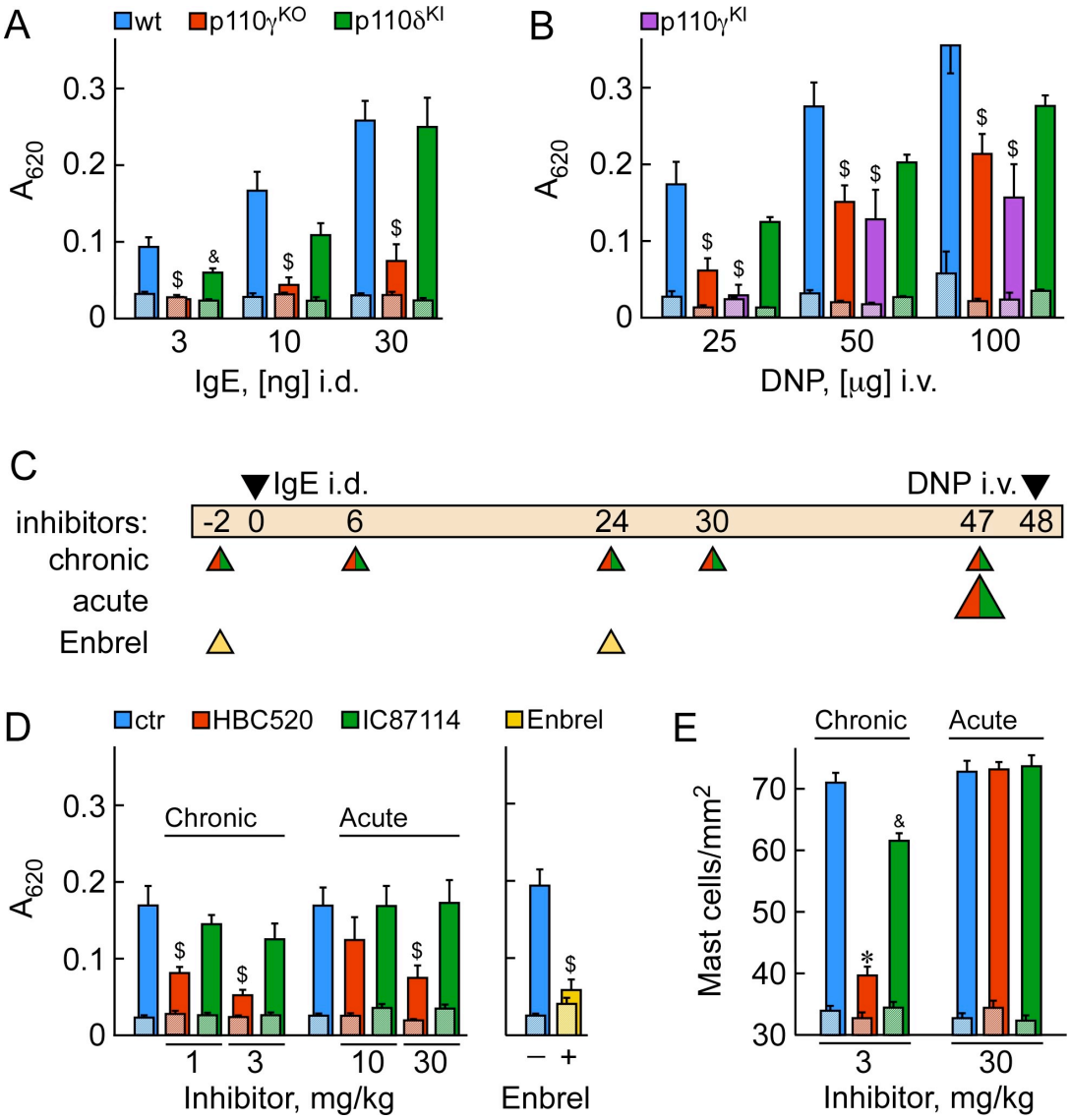

Figure 7. Contribution of p110γ and p110δ to vascular permeability *in vivo*. (A) wt, $p110\gamma^{KO}$ and $p110\delta^{DA}$ mice were sensitized with different doses of IgE (filled bars) or PBS (dashed bars) and i.v. injected 48 hrs later with 25 μg DNP (n>5, mean ± SEM, $ p<0.01 compared with wt). (B) Wt, $p110\gamma^{KO}$, $p110\gamma^{KR}$ and $p110\delta^{DA}$ mice were sensitized with 10 ng IgE (filled bars) or PBS (dashed bars) and i.v. injected 48 hrs later with different doses of DNP (n>5, mean ± SEM, $ p<0.01 compared with wt). (C) Protocol of chronic (1 or 3 mg/kg), acute (10 or 30 mg/kg) inhibitors or Enbrel (10 mg/kg) treatment. The chronic treatment was performed at -2, 6, 24, 30 and 47 hrs. For acute treatment, mice received a single dose at 47 hrs administrated p.o. Enbrel was given at -2 and 24 hrs. (D) Wt mice were sensitized with 10 ng IgE or PBS and i.v. injected 48 hrs later with 25 μg DNP and treated with p110 inhibitors (left panel). Wt mice were also treated with Enbrel (right panel) (n>5, mean ± SEM, $ p<0.01 compared with control). (E) Wt mice received chronic and acute inhibitor treatments and were killed 48 hrs after IgE or PBS i.d. injections. MCs were stained with Toluidine Blue O and counted using a light microscope (n>3, mean ± SEM, * p<0.001 compared with control).

Committed step of mast cell recruitment, activation of endothelia and/or PI3K in mast cells?

As mast cell recruitment and the progression of PCA seem to go hand in hand, we wanted to elucidate if the PI3K-dependent activation of mast cells, or the activation of endothelial cells was crucial for the recruitment process. To evaluate these steps, TNF-α was injected intrascrotally to generate inflamed endothelia in the cremaster muscle vasculature. Here, intra vital microscopy was exploited to monitor the rolling and adhesion of fluorescently pre-labelled BMMCs with wild type, $p110\gamma^{KO}$ and $p110\delta^{DA}$ genotypes. It became apparent, that wild type and $p110\delta^{DA}$ BMMCs readily docked to endothelia, while the interactions of BMMCs devoid of p110γ showed an impaired adhesion *in vivo*. (Fig. 8A and B and movie_1). This implies that the GPCR/p110γ-dependent increase in adhesion – most likely by upregulation of α4β1 integrins – plays a central role in the recruitment process.

The IgE-triggered recruitment of mast cells can be blocked, however, by Enbrel (Fig. 8C) illustrating that the action of TNF-α is also crucial to provide endothelial docking sites. The involvement of FcεRI-activated tissue mast cells in the process is supported by the fact that neutralizing anti-IgE antibodies inhibited mast cell recruitment as efficiently as Enbrel, while isotype and anti-IL1β antibodies remained ineffective.

Altogether, the above results document that mast cell recruitment, acute degranulation and cytokine release can be modulated by the inhibition of p110γ and to a lesser extent by targeting p110δ, and that this strategy attenuates multiple p110γ–dependent steps in the evolvement of the allergic response from the blood stream to the IgE challenged tissue. As a further insight, chronic ablation of PI3K activity proved to be superior, which might allow the lowering of applied inhibitor doses and improve drug safety.

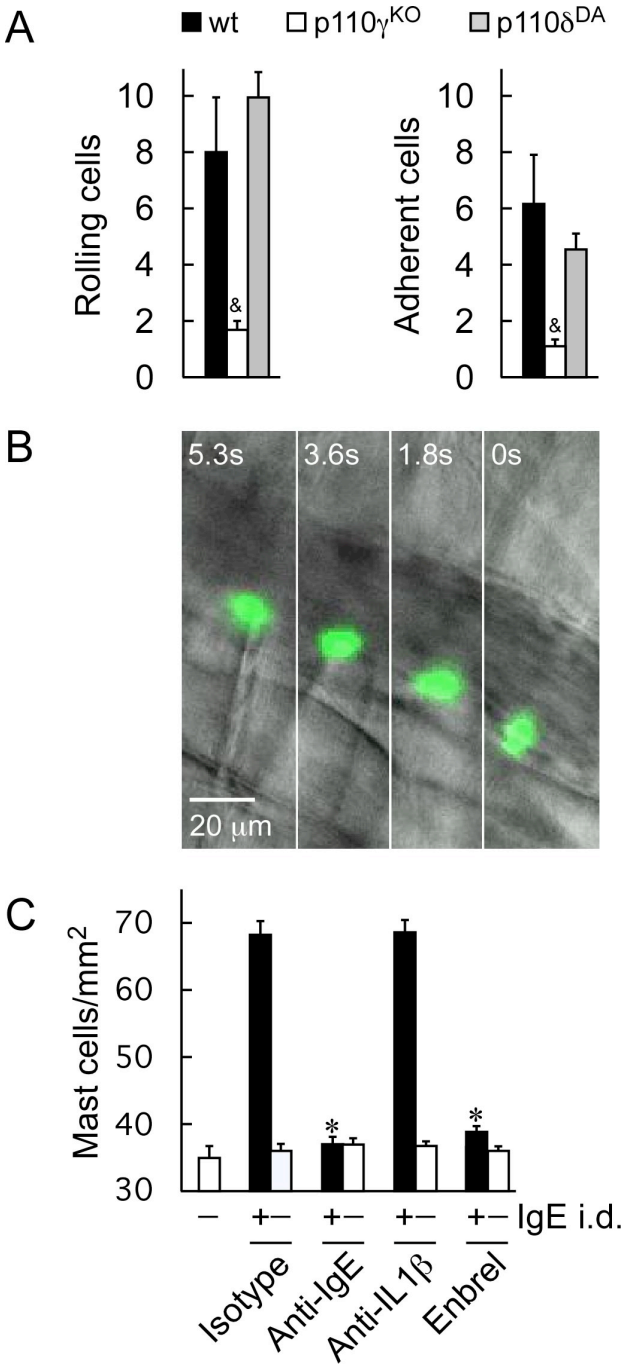

Figure 8. Mast cell recruitment upon stimulation. (A) 4 hrs after stimulation with TNF-α, the number of rolling (left panel) and firmly adherent cells (right panel) were quantified in the cremaster muscle using *in vivo* fluorescence microscopy (n=5; mean ± SEM; & $p<0.05$ compared with wt). (B) Rolling wt mast cell (in green) in the blood stream. (C) Wt mice received i.p. injection with 10 mg/kg rat IgG isotype control, neutralizing anti-IgE, anti-IL-1β antibodies or Enbrel 12 hrs prior to IgE or PBS i.d. injections and were killed 24 hrs later. MCs were stained with Toluidine Blue O and counted using a light microscope (n>3, mean ± SEM, * $p<0.001$ compared with wt isotype treated).

5. Discussion

In BMMCs, p110γ is the major PI3K isoform operating downstream of GPCRs [16] whereas p110δ controls c-kit, the SCF receptor [12]. Which PI3Ks command the IgE/antigen-FcεRI pathway is controversial. In the present study, we report evidences that p110γ is required for signaling through FcεRI. *In vitro*, p110γ is important for BMMCs adhesion to FN with stimulation such as adenosine or IB-MECA but also IgE alone and IgE/antigen whereas the major role of p110δ in this adhesion process is through c-kit (Fig. 2A). Similar to IgE/antigen and SCF-induced adhesion [52,53], adenosine and IB-MECA-induced adhesion is mediated through α5β1 integrin (Fig. 2B, C). Importantly, we have then described a role for p110γ during BMMC adhesion to endothelia when cells are in presence of adenosine, IB-MECA or chemokines (Fig. 3A). Consistent with the results discussed above, the role of p110δ is limited to SCF-induced adhesion to HUVECs. *In vitro* BMMC migration assay also relates the central position of p110γ in this process. Indeed, adenosine, IB-MECA, IgE and IgE/antigen-induced migration are all impaired in the absence of p110γ. As shown before, only SCF-induced migration is reduced by p110δ inactivation (Fig. 2D). *In vivo* experiments demonstrate that p110γ is also essential for mast cell recruitment after IgE injection in the murine skin (Fig. 1). Indeed, it is unlikely that mast cell have proliferated in the short time notice or induced by IgE alone.

Mast cells contribute to defend the body against pathogens and parasites [54,55] and their activation also leads to allergic inflammatory reactions in response to antigen such as asthma, allergic rhinitis, eczema and anaphylaxis. In response to inflammation, mast cells produce and release various inflammatory mediators including histamine, leukotriene C_4, prostaglandin D_2, cytokines, chemokines and proteases [56,57]. TNF-α and IL-6 are secreted in wt BMMCs upon IgE/DNP and IgE/DNP plus adenosine stimulation. For the first time, we have shown that in the absence of p110γ, or wt treated with p110γ inhibitor, the cytokine secretion is closed to baseline observed with IgE sensitization. In comparison, the inactivation of p110δ results in a diminution of the secreted cytokines upon IgE/DNP stimulation [12] but is immediately "rescue" by addition of adenosine (Fig. 5A, B). TNF-α is important for VCAM-1/ICAM-1 upregulation. Indeed, the use of an α-TNF-α antibody or

Enbrel completely abrogates the effect of wt BMMC Sn on VCAM-1/ICAM-1 upregulation (Fig. 4C, D). The lack of TNF-α secretion in p110γKO BMMCs explains why the level of surface adhesion molecules on endothelia is not increased in the presence of $p110\gamma^{KO}$ BMMC Sn (Fig. 4A, B). Moreover, Enbrel totally abolishes mast cell recruitment *in vivo* (Fig. 5C). These results demonstrate conclusively that the release of TNF-α-mediated by p110γ is important both *in vitro* and *in vivo*.

The SCF and IgE/antigen signaling have been analyzed by PKB phosphorylation, a downstream effector of PI3Ks. Unsurprisingly, PKB phosphorylation upon SCF stimulation is completely abrogated by inactivation of p110δ compare to wt or $p110\gamma^{KO}$ BMMCs (Fig. 6A). However, IgE/antigen signaling is impaired in absence of p110γ but not when p110δ is inactivated suggesting that the loss of p110γ is mainly responsible for signaling through FcεRI (Fig. 6B). Consistent with these results, BMMC degranulation is strongly reduced in absence of p110γ whereas the level of degranulation in $p110\delta^{DA}$ BMMCs is similar to those obtained with wt BMMCs (Fig. 6C). However, Ali et al., [12,24] have shown a reduction in PKB phosphorylation and degranulation upon IgE/DNP when p110δ is inactivated. Others have studied c-kit and FcεRI signaling using $p85\alpha^{-/-}$ BMMCs [58], the most abundant class IA PI3Ks adaptor subunit. It has been found that the loss of p85α reduced the expression level of class IA PI3K catalytic subunits p110α, p110β and p110δ. Lu-Kuo *et al.* have demonstrated that p85α is essential for both degranulation and phosphorylation of PKB signals upon SCF stimulation whereas it is dispensable for FcεRI-induced degranulation and phosphorylation of PKB.

In vivo PCA experiments with different doses of IgE and DNP confirm the critical role of PI3Kγ (Fig. 7). Similar results have been obtained by Laffargue *et al.* using passive systemic anaphylaxis (PSA) experiments. Fukao *et al.* have described that the systemic anaphylaxis shock responses are intact with mice lacking p85α. In the same study, they also observed an impaired c-kit signaling in $p85\alpha^{-/-}$ BMMCs whereas the FcεRI-mediated degranulation is not affected. These and the results obtained in the present study are further evidences that p110δ is essential in c-kit signaling whereas p110γ is a key element of the FcεRI-induced *in vitro* and *in vivo* signaling.

Mast cells are known to be involved in the development of allergic inflammation. These diseases affect a very large number of the human population and include asthma, allergic rhinitis, atopic dermatitis, anaphylaxis and allergic eye disease. In all theses, mast cell numbers are increased, degranulation intensified and cytokines production induced [59]. As we have shown in figure 1, i.d. injection of IgE increases the number of dermal mast cell in wt but not in p110γ. The use of Enbrel also impaired the mast cell recruitment. In absence of p110γ, TNF-α and IL-6 secretion are diminished. In asthma, VCAM-1/ICAM-1 are expressed on airway smooth muscle cells and may be involved in retention of mast cells and others immune cells [60] and this study reveals the importance of p110γ and TNF-α in this process. These results indicate that both p110γ and TNF-α would be appropriate intracellular targets in the treatment of human allergic diseases.

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7. Supplementary Materials and Methods

Mast cell progenitor staining

BM from wt, p110γ^{KO} and p110δ^{DA} were isolated. After lysing the red blood cells with Tris-buffered ammonium chloride, bone marrow cells were stained with FITC-labeled antibodies specific for the following lineage markers: CD3 (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53.6.72), CD11b (clone M1/70), CD11c (clone N418), B220 (clone RA3-6B2), CD19 (clone 1D3), NK1.1 (clone PK136), Sca.1 (clone D7), TER119 (clone TER-119) and with PE-labeled FcεRI (clone MAR-1) and APC-labeled c-Kit (clone ACK2). All antibodies were a generous gift from Antonius Rolink. The cells were incubated 30 min at 4°C, washed and flow cytometry acquisition was performed with FACS Calibur (BD). The data were analyzed with Flowjo (Treestar).

BMMC stimulation

BMMCs were preloaded overnight with 100 ng/ml IgE, washed, re-suspended at 1x10^6 cells/ml, and stimulated with different DNP or SCF concentrations or 0.5 μM adenosine. BMMCs were incubated for 1 hr with inhibitors at 10 μM or 0.1% DMSO before stimulations.

***f*MLP Assay:**

Fresh human blood from donors was collected in 20 ml tubes containing 20 mg/ml EDTA/PBS anticoagulant. Blood was subjected to red blood cell sedimentation over 4% dextran/PBS, for 30 minutes, on ice. The leucocyte-containing supernatant was layered on a Ficoll-Paque™ (Amersham Pharmacia, Sweden) density gradient and centrifuged at 350g, for 20 minutes, at 18°C. The pellet containing the granulocytes was then lysed in cold water and the isotonic balance was restored by adding X2 PBS before centrifugating 8 minutes at 350g and at 4°C. The pellet of granulocytes (neutrophils purity > 97%) was resuspended at 4.10^6 cells/ml in HBSS + 0.1% BSA and left on ice. 50 μl of neutrophils were added to the Costar 3912 microtitre plates (2.10^5 cells/well). 50 μl of inhibitors were added to the wells and incubated 15 minutes, at 37 °C. 50 μl of lucigenin was dispensed to all wells (final concentration = 100μM). The plate was then placed inside the Microluminat LB 96P and 100

μl of 5 μM fMLP was dispensed automatically by the luminometer into selected wells. 100 μl of PBS/1mM $CaCl_2$/1mM $MgCl_2$ was added manually to control wells containing 'unstimulated' cells. Total chemiluminescence and the temporal profile of each well were measured simultaneously for 6 minutes.

Human B cell proliferation assay:

B cells were purified from donors blood according to the protocol supplied by Dynal (Dynal Product code: 113.13). Isolated human B cells ($0.5x10^5$/well) were incubated with rabbit anti-human IgM antibody (10μg/mL final concentration; Stratech scientific Ltd: 309-006-043) in RPMI, 10% FCS, 1% MEM non-essential amino acids, 1μL ^{3}H-thymidine (1μCi/well) in 96-well plate for 48 hours. Incorporated thymidine was measured following extensive washing using a Packard TopCount.

8. Supplementary Figures and Tables

Table S1: *In vitro* IC_{50} values of class I PI3K specific inhibitors, [μM].

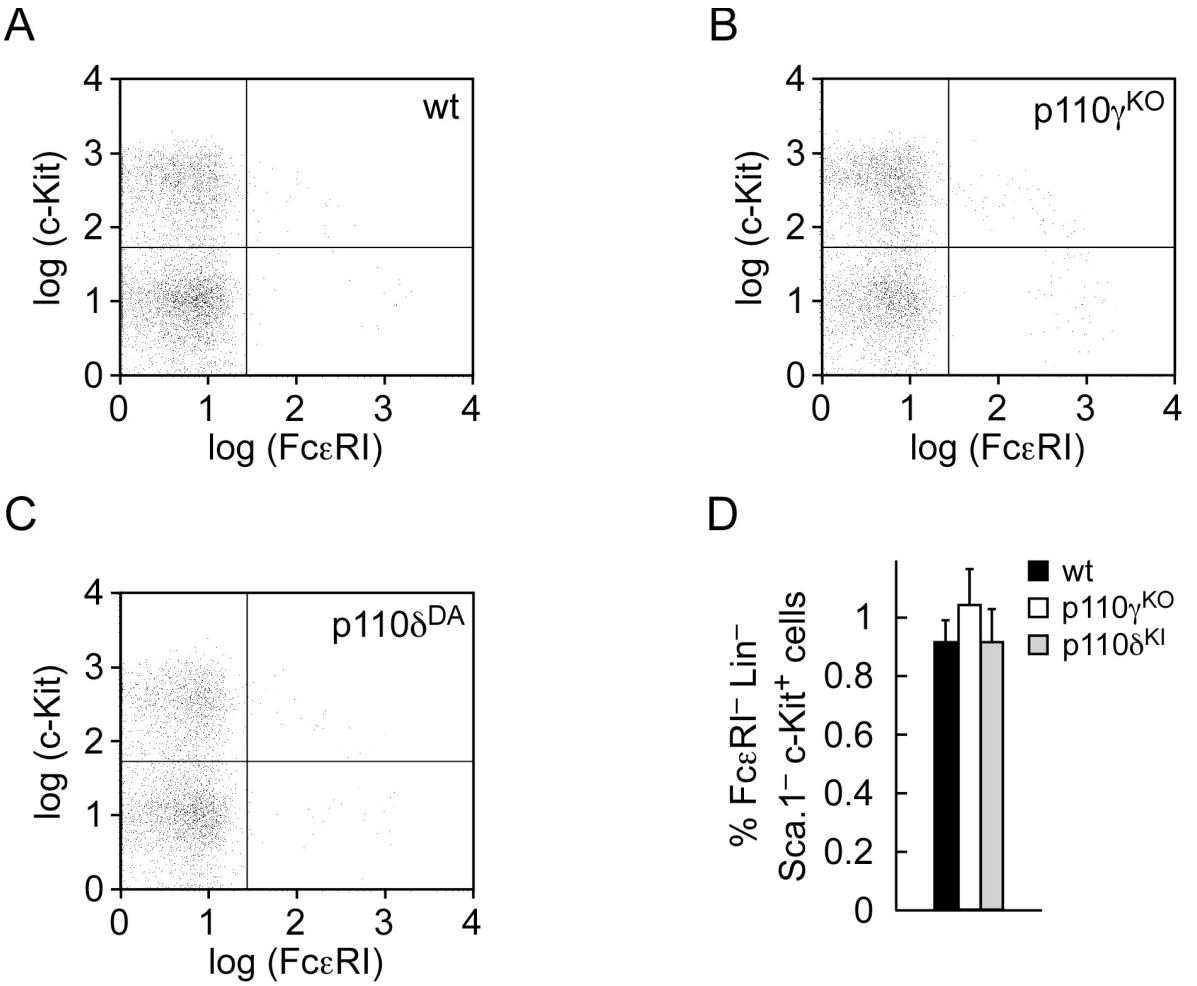
PI3K isoform	TGX-221	A252424	HBC520	IC87114
p110α	0.78[50]-5[61]	0.071[50]-0.94[51]	1.8[$]	>100[49]-200[62]
p110β	0.005[61]-0.01[50]	1.2[50]-20[51]	0.25[$]	16[62]-75[49]
p110γ	3.2[50]- >10[61]	0.012[50]-0.03[51]	0.09[$]	29[49]-61[62]
p110δ	0.065[50]-0.1[61]	0.19[50]-20[51]	0.75[$]	0.13[62]-0.5[49]

Data were compiled from: [61], [50], [51], [62], [49] and [$]present work.

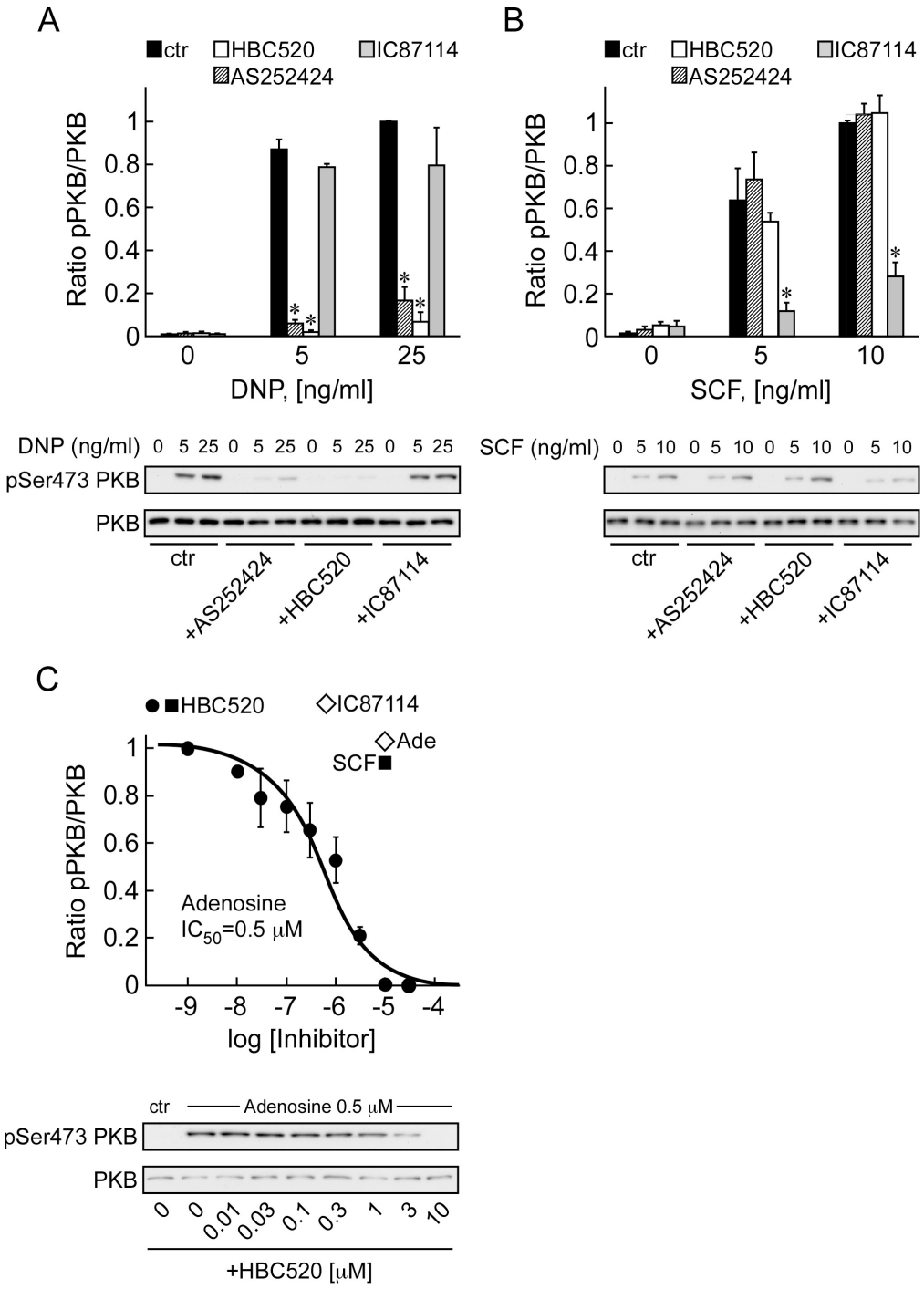
Table S2: Cell based IC_{50} values (μM) of class I PI3K specific inhibitors.

AS252424	HBC520	IC87114	*In vivo* response
8[50]	0.3[$]	1.9[$]	*f*MLP-induced response
	1.6[$]	0.05[$]	Human B cell proliferation
	0.5[$]	3.6[24]; >10[$]	Adenosine-induced pPKB/Akt
4.29[24]; >10[$]	>10[$]	0.27[24]	SCF-induced pPKB/Akt

Data were compiled from: [50] *f*MLP-induced PtdIns(3,4,5)P_3, [24] and [$]present work (the *f*MLP-induced response here was respiratory burst measured as indicated in supplementary materials).



Supplementary figure S1: BM from wt, p110γ^{KO} and p110δ^{DA} mice were isolated. Cells negative for Lin and Sca.1 were gated for FcεRI^{-} and c-Kit^{+}. Representative FACS plot of mast cell progenitor cells in the bone marrow of wt (A), p110γ^{KO} (B) and p110δ^{DA} (C) mice. (D) Percentage of mast cell progenitors was determined by staining for FcεRI^{-}, Lin^{-}, Sca.1^{-} and c-kit^{+} cells (n=3, mean ± SEM).



Supplementary figure S2: Wt BMMCs were incubated overnight with 100 ng/ml IgE, starved in medium containing 2% FCS for 3 hrs, treated with 10 μM of inhibitors during the last hour and (A) stimulated with different DNP concentrations (n=3, mean ± SEM, * $p<0.01$ compared with control) or (B) different SCF concentrations (n=3, mean ± SEM, * $p<0.05$ compared with control). (C) Wt BMMCs were starved in medium containing 2% FCS for 3 hrs, treated with different HBC520 concentrations during the last hour and stimulated with 0.5 μM adenosine. The ratio pPKB/PKB have been quantified using ImageJ and IC_{50} was determined (n=3, mean ± SEM).

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CONCLUSIONS

The work presented in this thesis aimed to identify and evaluate the role of PI3Kγ and PI3Kδ in mast cell activation and recruitment *in vitro* and *in vivo*. Indeed, previous studies have shown that PI3Ks are relevant enzymes in mast cell-mediated allergic response, but the importance of PI3Kγ and PI3Kδ is controversial ((Laffargue et al., 2002) vs (Ali et al., 2008)).

For the first time, PI3Kγ was shown to drive chemokine-induced BMMCs adhesion to endothelia, a process mediated by α4β1 integrin. Moreover, functional PI3Kγ is required for upregulation of VCAM-1/ICAM-1 on endothelial cells through TNF-α produced by mast cells. We also demonstrated that PI3Kγ is indispensable in adenosine, IB-MECA, IgE and IgE/antigen-induced mast cell adhesion to fibronectin.

While it remains uncontested that PI3Kδ is essential for signaling downstream of the c-kit receptor, its role in the IgE/antigen pathway is minimal in our hand. Indeed, PI3Kδ is not necessary neither for IgE/antigen-mediated mast cell degranulation nor for PKB phosphorylation. On the contrary, the main isoform involved in signaling downstream of the FcεRI receptor is PI3Kγ.

In vitro, it has been reported that BMMCs migration is induced by SCF activation in a PI3Kδ-dependent manner (Ali et al., 2004) whereas adenosine, IgE or IgE/antigen stimulations required functional PI3Kγ (Kitaura et al., 2005). We were able to confirm the role of each lipid kinase in migration assay. We then performed *in vivo* experiments to evaluate the relevance of these *in vitro* observations.

For the first time, we demonstrated that in tissues, mast cells are attracted to IgE by a mechanism determined by PI3Kγ. Indeed, mast cell recruitment is completely abrogated in absence of PI3Kγ, but not affected by the loss of PI3Kδ function, suggesting that PI3Kδ is irrelevant in acute mast cell recruitment. Our point of view is also strenghten by the fact that only PI3Kγ and not PI3Kδ inhibitors prevent mast cell accumulation. These results are in agreement with the fact that PI3Kγ is the major isoform involved in mast cell adhesion on fibronectin upon IgE stimulation.

Passive cutaneous anaphylaxis experiments also confirmed the prominent role of PI3Kγ. Indeed, both genetic and pharmacologic inhibition severely attenuates the anaphylactic shock. These findings also reinforce the data obtained *in vitro* with BMMCs degranulation. The *in vivo* allergic response was also modulated by TNF-α as Enbrel protects mice from passive cutaneous anaphylaxis confirming the important role of TNF-α. Intravital microscopy experiments also support these findings since only PI3Kγ, but not PI3Kδ, is required for rolling and firmly adherence of mast cells in the cremaster muscle after TNF-α stimulation. These results are supported by the fact mice treated with Enbrel did not display any mast cell recruitment to tissues. Altogether, modulation of PI3Kγ activity affects mast cell activation, recruitment and allergic responsiveness.

The results of the present study indicate that the IgE levels might correlate with mast cell numbers *in vivo*. Indeed, a recent study supports this evidence and reports that mice subjected to inhalation of *Aspergillus fumigatus (Af)* displayed a vigorous IgE response accompanied by a dramatic expansion of mast cells in trachea, bronchus and spleen (Mathias et al., 2009). Previously, it has been shown that IgE acts as a positive regulator of FcεRI on mast cell surface (Yamaguchi et al., 1997) and indeed the IgE receptor is upregulated on lung mast cells of wt mice following *Af* exposure (Mathias et al., 2009). It would be interesting to assess whether the loss of PI3Kγ is able to prevent the FcεRI upregulation in our model. In addition, IL-5-derived mast cells is increased in wt mice after *Af* exposure leading to an increased eosinophilia (Mathias et al., 2009). As PI3Kγ is required for mast cell recruitment, it would make sense to compare the cytokine profile in tissues from wt, $p110\gamma^{KO}$ and $p110\delta^{DA}$ exposed to IgE or IgE/antigen leading us to a better understanding of the mechanism.
IgE plays a central role in allergy as its level influences the mast cell number located within tissues. IgE is produced by B cells through a class-switch recombination (CSR) process that required expression of activation-induced cytidine deaminase (AID). Interestingly, inactivation of p110δ leads to increased IgE production and IgE expression on B cells probably because p110δ-inactive B cells expressed 2 fold-more AID transcripts than wt B cells (Zhang et al., 2008). These results raise new questions. Indeed, if the IgE level correlate with the number of mast cells, it would explain why the mast cell accumulation is not

reduced in p110δ mice. Additionally, it would be interesting to know if the IgE level in absence of PI3Kγ is reduced which could explain the reduction in mast cell recruitment.

Studies in allergic and atopic patients have demonstrated an increased mast cell number in tissues leading to re-evaluate mast cell as a crucial effector cell in allergic disease. Treatments of allergic diseases aim mainly to block mast cell degranulation and mediator release or to prevent the binding of IgE onto mast cells but none act on mast cell expansion in tissues. Thus, new therapeutic strategy can be envisaged like a PI3Kγ inhibitor which would prevent the accumulation of mast cell number in tissues, reducing at the same time the degranulation and release of inflammatory mediators and therefore the recruitment of other leukocytes.

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APPENDIX

Work discussed in the present thesis

PI3K Modulates Mast Cell Activation in Allergy
– from Blood to Tissue

Collmann E, Bohnacker T, Marone R, Dawson J, Rehberg M, Krombach F, Owen C, Burkhart C, Hirsch E, Thomas M, Wymann M.
Manuscript in preparation.

Other participation

PI3Kγ adapter subunits define coupling to degranulation and cell motility by distinct PtdIns(3,4,5)P_3 pools.

Bohnacker T, Collmann E, Marone R, Calvez R, Hirsch E, Wymann M.
These results have been accepted for publication in Science Signaling.

CELL BIOLOGY

PI3Kγ Adaptor Subunits Define Coupling to Degranulation and Cell Motility by Distinct PtdIns(3,4,5)P_3 Pools in Mast Cells

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Phosphoinositide 3-kinase γ (PI3Kγ) plays a major role in chronic inflammation and allergy. It is a heterodimer of a catalytic p110γ subunit and an adaptor protein, either p101 or the p101 homolog p84 (p87^{PIKAP}). It is unclear whether both PI3Kγ complexes specifically modulate responses such as chemotaxis and degranulation. In mast cells, the p84-p110γ complex synergizes with immunoglobulin E (IgE)– and antigen-clustered FcεRI receptor signaling and is required to achieve maximal degranulation. During this process, PI3Kγ is activated by ligands of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs), in particular adenosine receptors, through autocrine and paracrine pathways. Here, we show that p110γ needs p84 to relay signals from GPCRs to formation of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], phosphorylation of Akt, migration of cells, and synergistic adenosine-enforced degranulation. Furthermore, the absence of adaptor subunits could not be compensated for by increased p110γ abundance. Differentiated, p110γ null cells also lost adaptor proteins. Complementation of p110γ null mast cells with p101 and p110γ restored the activation of Akt and cell migration, but failed to support degranulation. Lack of degranulation was attributed to a change in the spatiotemporal localization of PI3Kγ-derived PtdIns(3,4,5)P_3; although both p84-p110γ and p101-p110γ complexes initially deposited PtdIns(3,4,5)P_3 at the plasma membrane, p101-p110γ–derived PtdIns(3,4,5)P_3 was rapidly endocytosed to motile, microtubule-associated vesicles. In addition, p84-p110γ, but not p101-p110γ signaling was sensitive to disruption of lipid rafts. Our results demonstrate a nonredundant function for the p101 and p84 PI3Kγ adaptor proteins and show that distinct pools of PtdIns(3,4,5)P_3 at the plasma membrane can elicit specific cell responses.

INTRODUCTION

Phosphoinositide 3-kinases (PI3Ks) are implicated in cancer (*1*–*4*) and inflammation (*5*, *6*), and their net activity can modulate the transition from normal cell functions to the progress of disease. Regulatory subunits translocate the 110-kD catalytic subunits—consisting of class IA PI3K members p110α, β, and δ and class IB p110γ—to their substrate phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] at the plasma membrane. For class IA PI3Ks, it is well documented that both Src homology 2 (SH2) domains of p85-like regulatory subunits (encoded by *PIK3R1*, *PIK3R2*, and *PIK3R3*) direct the PI3K complexes to phosphorylated Tyr-X-X-Met motifs on receptor protein tyrosine kinases or their substrates (*5*, *7*) and that interactions between p85 and p110 regulate enzyme activity (*8*, *9*). In spite of this knowledge, the physiological consequences of genetic ablation of regulatory p85 subunits have often been surprising. Whereas targeting of p85α (by deletion of exon 1 of *PIK3R1*) attenuated the function of PI3K downstream of the B cell receptor as expected (*10*–*12*), loss of p85α or p85β increased insulin signaling (*13*–*15*). Due to the multilevel complexity of the PI3K network, however, a full description of the physiological roles of the regulatory subunits of PI3Ks is far from being complete.

The sole member of the class IB family, p110γ (encoded by *PIK3CG*) operates downstream of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) (*5*, *16*) and is activated by βγ subunits of G proteins (Gβγ) in vitro (*17*, *18*). The catalytic subunit p110γ forms heterodimers with either of the adaptor subunits p101 (encoded by *PIK3R5*) (*18*) and a p101 homolog, p84 (also called p87^{PIKAP}, encoded by *PIK3R6*) (*19*, *20*). N- and C-terminal sites of p110γ bind to Gβγ subunits directly (*21*), but p101 and p84 are required for efficient Gβγ-stimulated phosphorylation of PtdIns(4,5)P_2 in vitro (*19*, *22*, *23*). The physiological importance of p101 was demonstrated convincingly in p101 null mice, in which GPCR ligands failed to trigger neutrophil chemotactic responses, whereas the induction of neutrophil NADPH oxidase was not affected (*24*), even though both responses are dependent on PI3Kγ (*16*). The p101 and p84 adaptor subunits perform equally well in vitro (*19*), but a physiological role for p84 is yet to be defined. It is still unclear whether p84 and p101 have overlapping or nonredundant functions in vivo.

We have therefore addressed the requirement and actions of PI3Kγ adaptor proteins in the physiologic context of mast cell activation, which provides a superb model system in which to study the function of PI3Kγ. In mouse mast cells, PI3Kγ signals downstream of A3 adenosine receptors (A3ARs) and drives cell migration (*25*), as well as synergistically enhancing IgE- and antigen-mediated degranulation in vitro and IgE- and antigen-induced passive systemic anaphylaxis in vivo (*26*). In this context, adenosine signaling through GPCRs is well known for activating mast cells in atopic, but not normal, patients (*27*, *28*).

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The clustering of FcεRI receptors through antigen-IgE complexes triggers a protein tyrosine kinase cascade that leads to the phosphorylation of immunoreceptor tyrosine–based activation motifs (ITAMs) on FcεRI receptor chains, multiple tyrosines on linker of activated T cells (LAT), non–T cell activation linker [(NTAL), also known as linker for activation of B cells (LAB), also known as LAT2] (*29*), and Tyr-X-X-Met motifs on growth factor receptor–bound protein 2 (Grb2)–associated binding protein 2 (Gab2) (*30*). Class IA PI3Ks, and in particular p110δ (*31*), thus produce a first wave of phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] as a consequence of the clustering of FcεRI receptors. This signal is, however, quenched by the action of the SH2 domain–containing inositol 5′-phosphatase (SHIP), which degrades PtdIns(3,4,5)P_3 to PtdIns(3,4)P_2 (*32*). In this context, GPCR-triggered activity of PI3Kγ delivers a superimposed pulse of PtdIns(3,4,5)P_3, which is integrated with the FcεRI protein tyrosine cascade and produces a full-scale activation and degranulation response. Its exclusive dependence on PI3Kγ makes this an attractive model in which to unravel the functions of the PI3Kγ adaptor proteins.

Here, we show that differentiated hematopoietic cells that lack the p110γ catalytic subunit also lose their PI3Kγ adaptor proteins, which was exploited to produce mast cells devoid of both p110γ and p84. Complementation assays showed that p110γ could not operate without its adaptor proteins. Moreover, p101 and p84 had nonredundant functions in a physiological context and produced distinct pools of PtdIns(3,4,5)P_3 with different fates, sensitivities to depletion of cholesterol, and capacities to promote release of mast cell granules. PI3Kγ adaptor subunits thus controlled spatiotemporal signaling of p110γ catalytic subunits to yield diverse physiologic outputs. Because PI3Kγ is an important component in chronic inflammation and allergy, a full understanding of its mechanism of activation might open previously unknown avenues of context- and tissue-specific targeting of PI3Kγ. Spatial organization might also apply to mammalian p85 class IA PI3K adaptor proteins, which have physiologically poorly explored interaction domains, such as the SH3 and breakpoint cluster region homology (BH) domains (*7*).

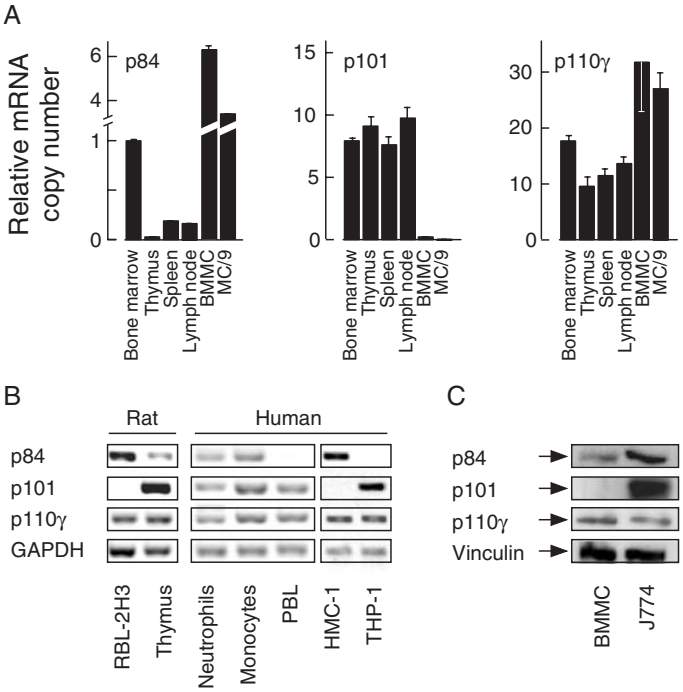

Fig. 1. Tissue distribution of p110γ and its adaptor subunits. (**A**) Relative copy numbers of mRNAs for p110γ, p84, and p101 were assessed by qPCR in the indicated murine tissues (for additional tissues, see fig. S1), as well as in BMMCs and a mouse mast cell line (MC/9). Data shown represent mean ± SEM of more than three experiments. All values are normalized to the copy number of p84 mRNA in BM. (**B**) p84, p101, p110γ, and GAPDH (internal standard) mRNAs were amplified from the RBL-2H3 and HMC-1 mast cell lines and the indicated rat and human cells and tissues by RT-PCR, separated by agarose gel electrophoresis, and visualized with ethidium bromide. (**C**) p101 protein is undetectable in murine BMMCs. Total lysates of murine BMMCs and J774 cells were subjected to SDS-PAGE and the indicated proteins were detected by Western blotting. (**D**) p84 protein is stabilized in the presence of p110γ. Bone marrow (BM), macrophages (MØ), splenocytes (Spleno), neutrophils (NØ), and mast cells (BMMC) from WT mice (WT), mice deficient in p110γ (KO), or mice with a catalytically inactivated p110γ (Lys^{833}→Arg mutation; KR) were probed for the presence of p110γ and p84 proteins by Western blotting. Blots in (B) to (D) are representative of more than three experiments each.

RESULTS

Mast cells: a model in which to study the functions of PI3Kγ adaptor proteins

To evaluate and compare the tissue distribution of p84-p110γ and p101-p110γ complexes, we measured the abundance of messenger RNAs (mRNAs) for mouse p110γ, p84, and p101 by quantitative polymerase chain reaction (qPCR) assays. mRNAs for p110γ, p84, and p101 were detected at high abundance in murine hematopoetic tissues and cells (Fig. 1A and fig. S1), corresponding to the reported role of PI3Kγ in the recruitment of leukocytes (*16*) and full-scale activation of mast cells (*26*). In all tissues investigated, p110γ mRNA correlated with the presence of that of at least one of the adaptor subunits, supporting the importance of the regulators to the function of PI3Kγ. The mRNA for p101 was prominently expressed in bone marrow (BM), thymus, spleen, and lymph nodes but was at low abundance in BM-derived mast cells (BMMCs) (see Fig. 1C for protein abundance) and mast cell lines of mouse (MC/9), rat (RBL-2H3), and human (HMC-1) origin. In contrast, the abundance of p84 mRNA was high in mast cells (Fig. 1, A and B). Similarly, we examined the abundance of PI3Kγ subunit proteins in hematopoietic cells derived from wild-type (WT) mice, p110γ null mice (KO) (*16*, *33*), or genetically targeted mice with catalytically inactive p110γ (KR, which contains a Lys^{833}→Arg mutation) (*34*). In BM samples, the abundance of p84 protein was not affected by manipulations of the p110γ locus (Fig. 1D). However, the abundance of p84 was lower in differentiated cells such as mast cells, macrophages, splenocytes, and neutrophils from KO mice than from WT mice, whereas the abundance of p84 was normal in cells from KR mice (Fig. 1D and fig. S2). This implies that the stability of p84 was dependent on the presence of p110γ but not on its kinase activity. That other, yet unexplored mechanisms control the abundance of

PI3Kγ adaptor proteins is illustrated by the stability of p84 in BM samples from KO mice and the lower abundance of p84 mRNA in KO BMMCs compared to that in WT BMMCs (fig. S2C).

Because BMMCs from KO mice lack both adaptor and catalytic subunits, we used them for complementation assays and functional studies. We first explored the physiological output of the p84-p110γ complex and then tested whether exogenous p101 could substitute for p84. Both adaptor proteins interact with p110γ and Gβγ subunits in vitro, although p101 sensitizes PI3Kγ for activation by Gβγ subunits at even lower concentrations of Gβγ subunits (*19*).

Combinations of WT or catalytically inactive (KR) p110γ proteins with either green fluorescent protein (GFP)–tagged p84 (GFP-p84) or p101 (GFP-p101) were introduced into KO BMMCs by nucleofection. Complementation of WT p110γ with GFP-p84 or GFP-p101 restored adenosine receptor signaling, as detected by the presence of phosphorylated Akt, but the isolated introduction of p110γ did not (Fig. 2A). Under these conditions, introduction of p110γ with or without GFP-p84 or GFP-p101 did not increase the abundance of endogenous p84 (fig. S3). As for WT BMMCs (*26*), adenosine-mediated phosphorylation of Akt in KO BMMCs reconstituted with either p110-adaptor complex was prevented by the PI3K inhibitor wortmannin (Fig. 2B) and by pretreatment with pertussis toxin (PTX) (fig. S4). The A3AR-specific agonist IB-MECA and agonists targeting all adenosine receptors (adenosine and NECA) stimulated phosphorylation of Akt to a similar extent independently of the PI3Kγ adaptor protein used, indicating that PI3Kγ adaptors did not alter adenosine receptor signaling (fig. S4).

A role for PI3Kγ in GPCR-induced chemotaxis has been established in various cell types (*16*, *24*, *35*, *36*). Mast cells migrate toward adenosine through activation of A3AR in a PI3Kγ-dependent, PTX-sensitive manner (*25*). When reconstituted with p110γ only, BMMCs derived from KO mice were unable to migrate toward adenosine, whereas reconstitution with WT p110γ and either hemagglutinin (HA)-tagged p84 (HA-p84) or p101 (HA-p101) resulted in the cells having an increased migratory capacity (Fig. 2C). Neither the presence of the individual adaptor proteins nor reconstitution with either adaptor protein and catalytically inactive p110γ (KR) restored

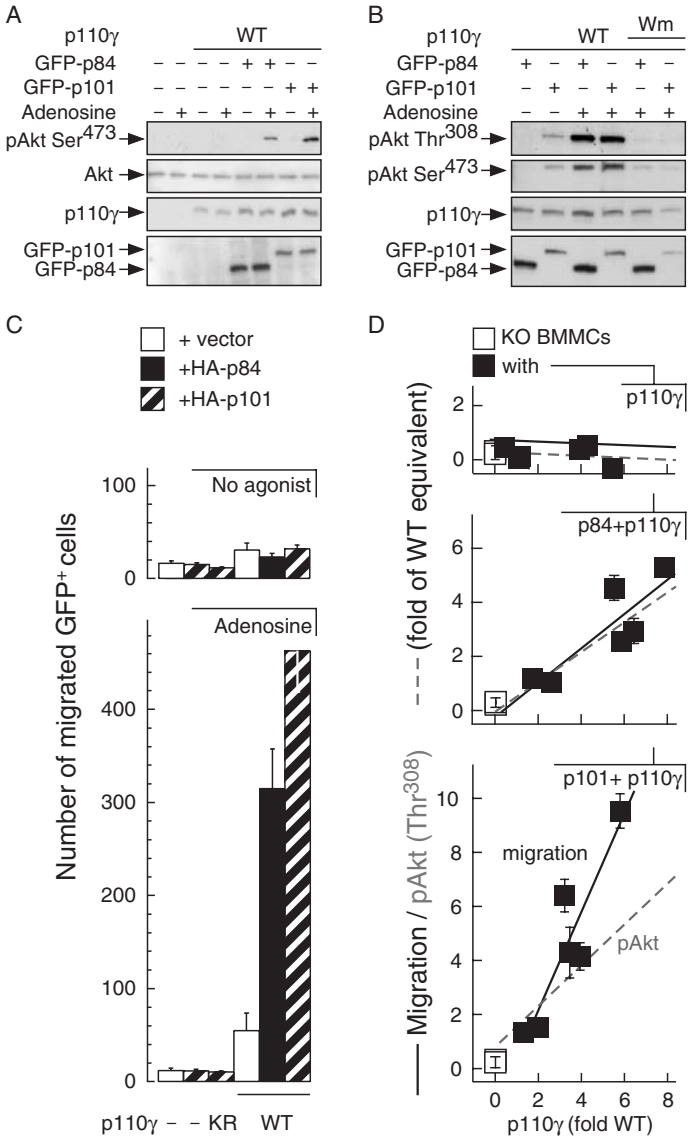

Fig. 2. Adenosine-induced phosphorylation of Akt and cell migration is restored in KO BMMCs by reconstitution with p110γ and either p84 or p101. KO BMMCs were transfected with plasmids encoding functional p110γ (WT) and either GFP-tagged p84 (GFP-p84) or p101 (GFP-p101). One day later, cells were starved in IL-3–free medium containing 2% FCS for 3 hours and stimulated with 2 μM adenosine for 3 min. (**A**) Phosphorylation of Akt at Ser^{473} was determined by analysis of Western blots of cell lysates with an anti–Akt-$pSer^{473}$. The abundance of p110γ was assessed with an anti-p110γ, whereas GFP-p84 and GFP-p101 were detected and their relative abundance was compared with an anti-GFP. One representative experiment of three is shown. (**B**) Stimulations performed as in (A) were tested for their sensitivity to 30 min of preincubation with 500 nM wortmannin (Wm). Phosphorylation of Thr^{308} of Akt was assessed in parallel with the appropriate antibody. (**C**) KO BMMCs were reconstituted with WT or KR p110γ and either HA-p84 or HA-p101 as indicated, as well as with GFP to select for transfected cells. Migration of GFP-positive BMMCs was assayed in Transwell chambers for 6 hours in the absence (top) or presence of 1 μM adenosine (bottom) in the lower well. Subsequently, GFP-positive cells were quantified. Data shown are the mean number ± SEM of migrated GFP-positive cells from $n = 3$ to 9 experiments. (**D**) Comparison of activation of Akt and migration of reconstituted KO BMMCs. KO BMMCs were transfected with plasmids encoding GFP, p110γ, and either HA-p84 or HA-p101 (as indicated, with variable DNA concentrations), whereas WT BMMCs were transfected with a plasmid encoding GFP as a control. Experiments to measure adenosine-stimulated Akt activity and cell migration were performed as described for (A) and (C), respectively. In parallel, the abundance of p110γ protein was determined by Western blotting. Cell responses (phosphorylation of Thr^{308} of Akt and cell migration) of complemented KO BMMCs are expressed as the fold difference to those of WT BMMCS and were correlated with the fold difference in the abundance of p110γ protein compared to that of WT BMMCs. Data shown are from $n = 3$ experiments, with two to three different DNA inputs in each experiment. Migration events were corrected by subtracting the number of migrating unstimulated cells to determine the number of cells that migrated in response to adenosine.

mast cell migration, showing that a functional p110γ-adaptor complex is required to sustain motility (Fig. 2C). This is underlined by the extent of adenosine-dependent phosphorylation of Thr^{308} of Akt and the efficiency of cell migration both being proportional to the amount of PI3Kγ complex present, whereas even when the abundance of p110γ was greater than six-fold higher than that of the endogenous protein in WT cells, the lack of adaptor proteins could not be compensated for (Fig. 2D).

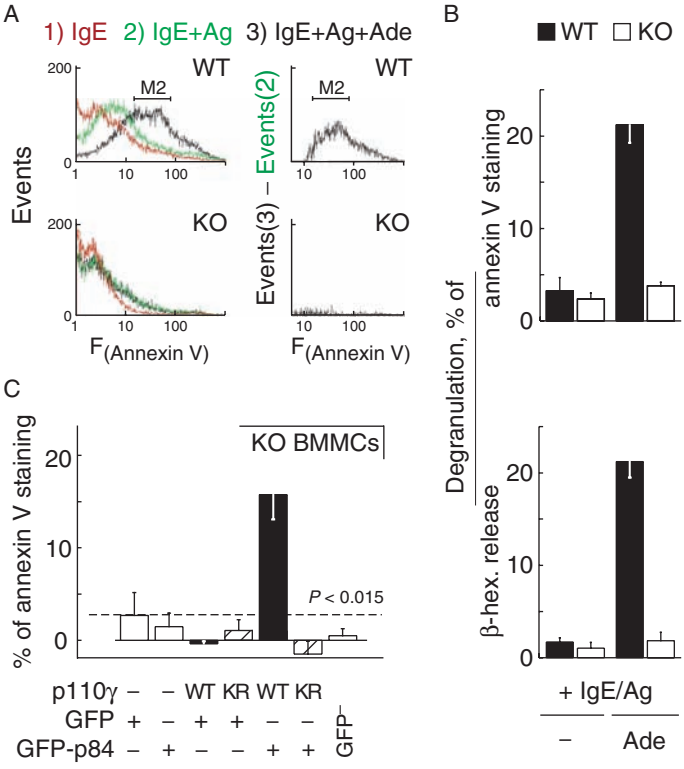

Fig. 3. Mast cell hyperreactivity requires p84. (**A** and **B**) Mast cell degranulation was assessed by annexin V staining and flow cytometry. WT and KO BMMCs were exposed overnight to anti-DNP IgE (100 ng/ml). (**A**) Left: Cells were stained with annexin V after loading with IgE (red), additional stimulation with DNP-HSA (1 ng/ml; Ag) for 20 min (green), or with DNP-HSA (1 ng/ml) and 2 μM adenosine for 20 min (black). Right: Adenosine-dependent activation is represented by the difference in histograms from [IgE+Ag+Ade] and [IgE+Ag] stimulations, given as Events(3) – Events(2). (**B**) Top: Quantification of the experiments shown in (A). Degranulation was calculated from events within region M2 after stimulation with IgE+Ag or IgE+Ag+Ade, as indicated. Data shown are the mean ± SEM of $n = 4$ experiments with two populations of BMMCs and are expressed as the percentage of annexin V–positive cells in the M2 gate (Degranulation, % of annexin V staining). Bottom: For comparison, β-hexosaminidase (β-hex.) release assays were performed under identical conditions. Data shown are the mean ± SEM of $n = 3$ experiments. (**C**) Restoration of adenosine-enforced degranulation in transfected KO BMMCs. KO BMMCs were transfected with expression plasmids for GFP or GFP-p84 in combination with p110γ (WT or KR). The adenosine-dependent component of degranulation in the reconstituted BMMCs is shown (ΔDegranulation; see Materials and Methods). Here, % of annexin V staining represents the difference [Events(3) – Events(2)] as explained for (A). Data shown are the mean ± SEM for $n > 3$ experiments. The dotted line sets $P < 0.015$ for comparison with p110γ:GFP-p84. The bar labeled with GFP^- denotes the degranulation of GFP-negative, electroporated, but nontransfected KO BMMCs, which served as an internal control. The setting of regions (GFP^+, M2, etc.) is explained in detail in fig. S5.

Nonredundant functions of PI3Kγ adaptor proteins

To investigate adaptor-dependent PI3Kγ signaling in the degranulation of mast cells, we adapted a flow cytometric assay first described by Demo *et al.* (*37*), which is based on the degranulation-induced, extracellular exposure of phosphatidylserine on the cell surface and the subsequent staining of cells with annexin V. Many groups (*37*–*40*) have established a direct correlation between the extent of release of β-hexosaminidase and the extent of annexin V binding in various contexts. With WT and KO BMMCs, we confirmed that the assay sensitively detected IgE- and antigen-mediated degranulation and we distinguished this from a full-scale response that involved costimulation of cells with adenosine (Fig. 3, A and B). In agreement with previous β-hexosaminidase assays, the stimulation of WT BMMCs with IgE and a low concentration of antigen resulted in little degranulation, which was even less so for KO BMMCs. As expected, adenosine-triggered hyperactivation of mast cells was completely abrogated in the absence of PI3Kγ (Fig. 3B). With the annexin V staining assay, we could separately analyze transfected and nontransfected cells through the detection of GFP or GFP fusion proteins by flow cytometry. GFP-negative cells served as an internal standard. When KO BMMCs were transfected with plasmids that encoded GFP-p84 and p110γ, responsiveness to adenosine was restored to that of WT BMMCs, and only the GFP–p84-p110γ complex could enhance degranulation after costimulation with adenosine. As was observed for phosphorylation of Akt and cell migration, the individual subunits GFP-p84 and p110γ, or the catalytically inactive p110γ-adaptor complex, failed to reconstitute adenosine-dependent degranulation of mast cells (Fig. 3C). These results underline that p110γ was incapable of signaling or contributing to degranulation in the absence of an adaptor subunit.

As shown above, p84 was indispensable for adenosine receptor–mediated PI3Kγ signaling that led to enhancement of IgE- and antigen-mediated degranulation (Fig. 3C). KO BMMCs reconstituted with GFP-p101 and WT p110γ, however, did not produce an adenosine-dependent increase in degranulation above that observed in response to IgE and antigen, whereas cells reconstituted with GFP-p84 and p110γ exhibited maximal degranulation (Fig. 4A). The abundance of p110γ, p84, and p101 proteins were also quantified. When the abundance of p110γ was normalized to that of the p84-p110γ complex, it was found that reconstitution of cells with p110γ alone resulted in a fivefold lower abundance of p110γ protein than that in cells reconstituted with both p110γ and p84. The p101 protein stabilized p110γ somewhat better than p84 did (Fig. 4B). There was no significant difference in the abundance of GFP-p84 and GFP-p101 (Fig. 4D), and both GFP-tagged adaptor proteins were localized to the cytosol (Fig. 4E). Because p101 translocates to the nucleus in the absence of p110γ (*41*), we assumed that cytosolic GFP-p101 was tightly bound to p110γ and was thus in a functional state. This is also in agreement with mediation of adenosine-induced activation of Akt and migration by the p101-p110γ complex. Similar results were obtained in experiments with cells reconstituted with GFP-fused p110γ and HA-tagged adaptor subunits (Fig. 4A). Consistent with the previous experiments, KO BMMCs reconstituted with HA-p84-GFP-p110γ complexes exhibited adenosine-dependent degranulation, whereas those reconstituted with HA-p101-GFP-p110γ complexes did not. In the latter experiments, fusion of GFP to the N terminus of p110γ stabilized the kinase when expressed alone, so that its abundance approached that observed in the presence of its adaptor proteins (Fig. 4C). Despite this increased abundance, GFP-p110γ alone was incapable of rescuing adenosine signaling in KO BMMCs.

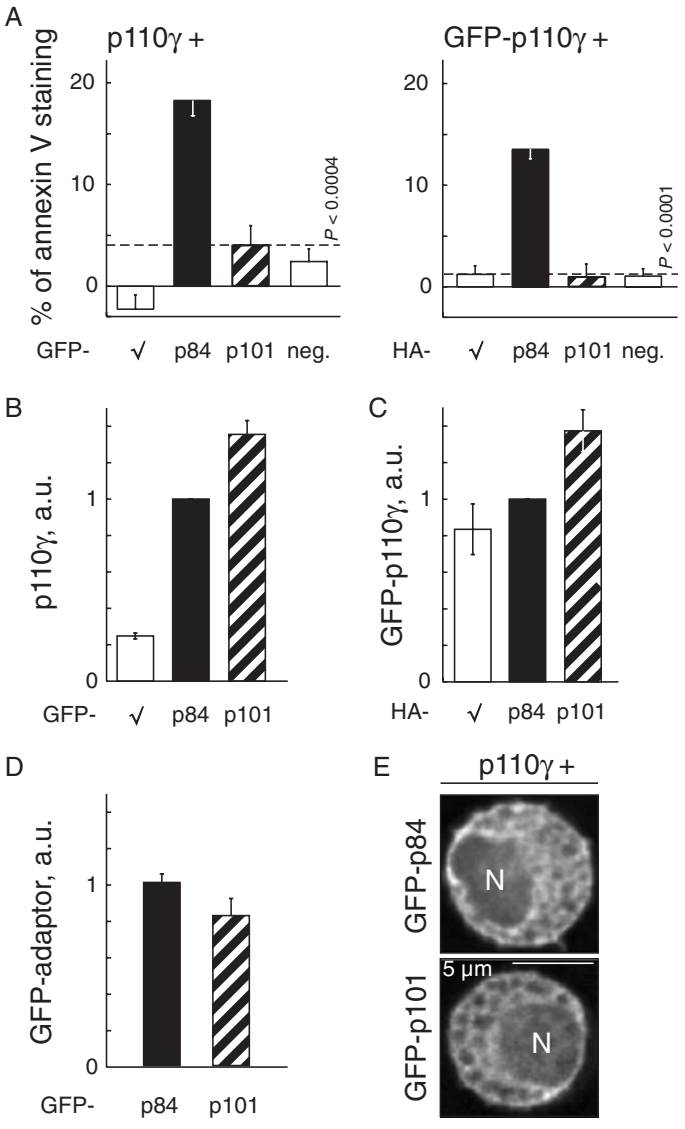

Fig. 4. The p101 adaptor protein does not compensate for p84 in degranulation responses. (**A**) Left: KO BMMCs were transfected with plasmids encoding p110γ in combination with plasmids encoding GFP (√), GFP-p84, or GFP-p101. Degranulation was measured after stimulation of cells with IgE and antigen with or without adenosine as in Fig. 3C. Data shown are the mean ± SEM for $n > 5$ experiments with $P < 0.0004$ for p84 compared to p101. "Neg." denotes the degranulation of GFP-negative, electroporated, but nontransfected KO BMMCs. Right: Experiments were performed as in the left panel but with GFP fused to p110γ instead of the adaptor proteins. (**B** to **D**) Quantification of restored p110γ and adaptor subunits on an Odyssey infrared-detection system. The abundance of p110γ is shown normalized to that of p110γ when coexpressed with p84. Data shown are the mean ± SEM of $n = 3$ experiments. (**B**) Quantification of data from the left panel of (A). (**C**) Quantification of data from the right panel of (A). (**D**) Quantification of the abundance of GFP-p84 and GFP-p101 proteins normalized to that of GFP-p84. Data shown are the mean ± SEM from $n = 2$ experiments. (**E**) Cellular localization of GFP-p84 and GFP-p101 in KO BMMCs reconstituted with either fusion protein and p110γ. Images are deconvoluted. N, nucleus.

Differences in p84- and p101-mediated localization of PtdIns(3,4,5)P_3

PI3Kγ-derived PtdIns(3,4,5)P_3 was visualized with the PtdIns(3,4,5)P_3-specific pleckstrin homology (PH) domain of Bruton's protein tyrosine kinase (Btk) (*42*) fused to GFP (PH_{Btk}-GFP). Although adenosine-triggered phosphorylation of Akt occurred with both types of PI3Kγ complex, the localization of PH_{Btk}-GFP was distinguishable depending on the adaptor protein used. Whereas adenosine caused the transient translocation of PH_{Btk}-GFP from the cytosol to the plasma membrane in cells containing HA–p84-p110γ complexes, in adenosine-treated BMMCs that contained HA–p101-p110γ complexes, PH_{Btk}-GFP displayed a vesicular distribution below the plasma membrane at later time points (Fig. 5A and movies m1 and m2). These endocytosed vesicles were separated from the plasma membrane and did not contain PtdIns(4,5)P_2, as visualized with a red fluorescent probe made from the PH domain of phospholipase C δ ($PH_{PLCδ}$-RFP) (Fig. 5B). Whereas the $PH_{PLCδ}$-RFP probe was constitutively localized at the plasma membrane, the membrane docking of PH_{Btk}-GFP required stimulation of the cells with adenosine, an active PI3Kγ complex, and an intact PH_{Btk} domain, because the Arg^{28}→Cys (R28C) mutation of PH_{Btk} blocked its translocation (Fig. 5, A and B). Therefore, PtdIns(3,4,5)P_3 was indeed present in the p101-dependent, internalized vesicles, whereas PtdIns(4,5)P_2 was excluded.

To test a correlation between the occurrence of intracellular PtdIns(3,4,5)P_3 and the abundance of p101, we performed an analysis of a population of cells to assess the extent of plasma membrane (rim) or internal, vesicular staining for PtdIns(3,4,5)P_3 (Fig. 6A). Within 20 s of stimulation with adenosine, cells that contained HA–p84-p110γ complexes displayed translocated PH_{Btk}-GFP at the plasma membrane and attenuated rim staining could be detected until 2 min later. Cells that contained HA–p101-p110γ complexes showed rim staining at early time points (<15 s), but intracellular speckles of PtdIns(3,4,5)P_3 also became visible. By 120 s after the addition of adenosine, most p101-containing cells displayed speckles, whereas p84-containing cells did not.

Live-cell microscopy was applied as an alternative method to monitor the relocalization of PtdIns(3,4,5)P_3. In single cells that contained HA–p84-p110γ complexes, translocation of PH_{Btk}-GFP to the plasma membrane was maximal at 40 to 50 s after the addition of adenosine and was maintained for less than 2 min after stimulation before moving from the rim region to the cytosol. In BMMCs that contained HA–p101-p110γ complexes, the intensity of fluorescence at the rim showed similar dynamics, but diminished because of endocytosis of PtdIns(3,4,5)P_3 (Fig. 6, B and C, fig. S6, and movies m3 and m4). Quantification of this process illustrated that PtdIns(3,4,5)P_3 was produced at the plasma membrane by both PI3Kγ complexes at the same rate, and that the pace of degradation of PtdIns(3,4,5)P_3 within the plasma membrane was comparable to the removal of PtdIns(3,4,5)P_3 by endocytosis (Fig. 6C). Costimulation of mast cells with IgE-antigen and adenosine dramatically prolonged and accentuated the localization of PH_{Btk}-GFP to the plasma membrane in cells containing p84-p110γ and delayed the onset of translocation and p101-p110γ-mediated endocytosis of PH_{Btk}-GFP to >2 min after stimulation with adenosine (Fig. 6, D and E, and movies m5 and m6). The prolonged retention times of PH_{Btk}-GFP at the plasma membrane might reflect a spatial synergy between p84- and class IA–derived PI3K activities.

Cytosolic, PtdIns(3,4,5)P_3-containing vesicles were mobile, and endocytosis of PtdIns(3,4,5)P_3 was followed by fast transcellular movements in a fraction of the speckles (fig. S6; see alternative analysis exemplified with movie m4). The curvature of the transport trajectories and the often perinuclear destination of the speckles suggested that a subset of the speckles was associated with microtubules. Indeed, pretreatment of BMMCs that contained HA–p101-p110γ with the microtubule disruptor nocodazole

attenuated the mobility of PtdIns(3,4,5)P_3-containing speckles, but not the initial endocytosis to a submembrane localization (fig. S6B and movies m8 and m9).

Disruption of cholesterol-rich domains affects the signaling of p84-p110γ, but not of p101-containing PI3Kγ

PI3Kγ complexes consisting of p101 were capable of driving a subset of cellular responses even though PtdIns(3,4,5)P_3 was endocytosed. The only response not promoted by p101 was degranulation, which requires the integration of signals derived from costimulation with IgE and antigen. Because cross-linked FcεRI receptors accumulate and signal in cholesterol-rich, plasma membrane microdomains called lipid rafts (*43–45*), we set out to test the sensitivities of both types of PI3Kγ complex to methyl-β-cyclodextrin (MβCD), a compound that depletes cholesterol from membranes. To monitor the process, we stained cells with fluorescently labeled cholera toxin subunit β (CTβ), a marker of lipid rafts. In untreated cells, CTβ showed a punctuate pattern, which was disrupted on exposure to MβCD (Fig. 7A). Of note, pretreatment with MβCD selectively attenuated adenosine signaling mediated by p84-p110γ in WT BMMCs (Fig. 7A) or by p84-p110γ in reconstituted KO BMMCs (Fig. 7, B and C). In contrast, p101-p110γ–mediated or interleukin-3 (IL-3)–stimulated phosphorylation of Akt was unaffected by disruption of lipid rafts (Fig. 7, B and C).

The above data add to the evidence that suggests that the PI3Kγ adaptors p84 and p101 serve a nonredundant function and can specifically diverge PI3Kγ signals downstream of GPCRs. Altogether, our results are compatible with a model in which both p110γ complexes integrate upstream signals from GPCRs and PTX-sensitive heterotrimeric G proteins, but differ in downstream processing toward a localized signal output.

Cells with endogenous p101 internalize PtdIns(3,4,5)P_3

Having shown the nonredundant role of PI3Kγ adaptor subunits in mast cells in which p84 is the predominant adaptor protein, we investigated translocation of PH_{Btk}-GFP in the J774 macrophage cell line, in which p101 is the predominant PI3Kγ adaptor protein (Fig. 1C and fig. S8C). It can therefore

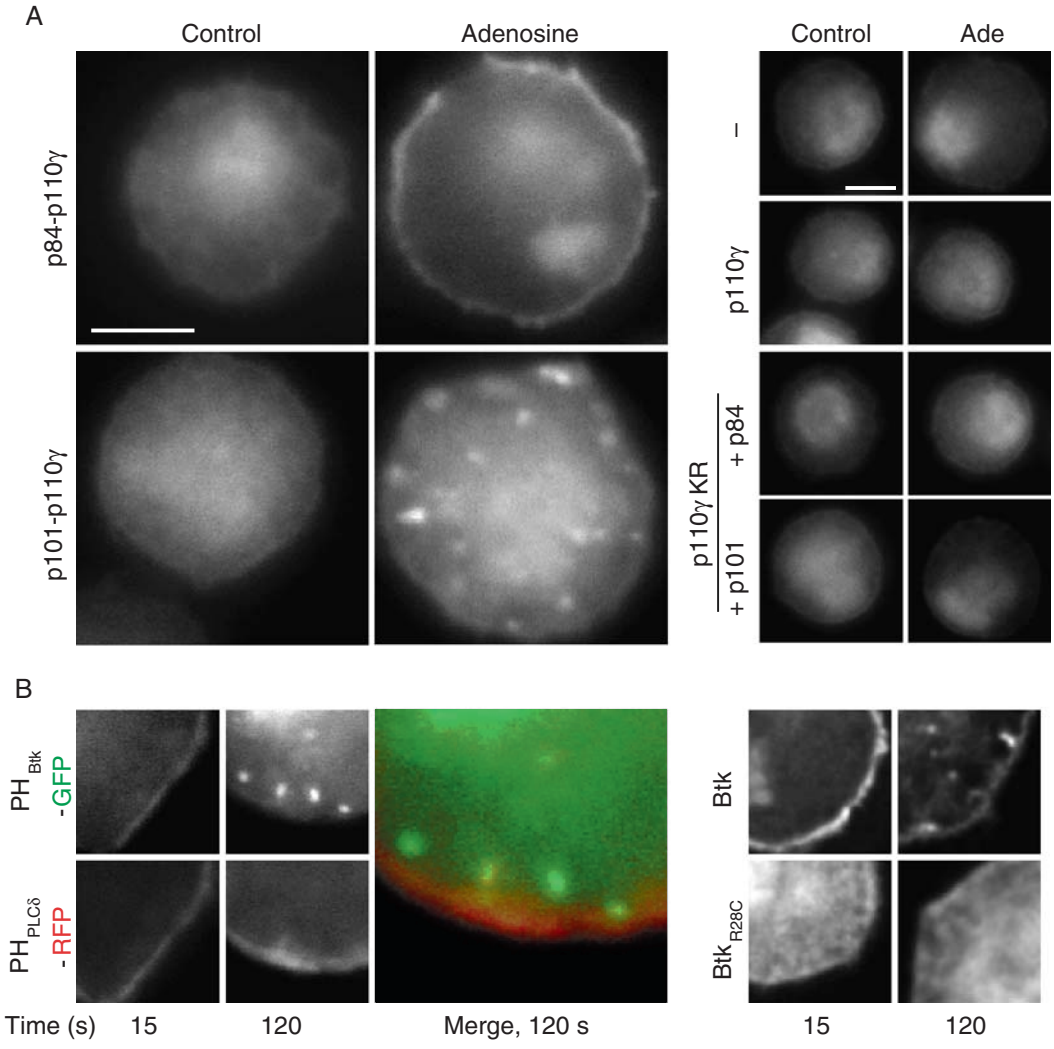

Fig. 5. Adaptor-specific, spatiotemporal differences in the localization of PtdIns(3,4,5)P_3. KO BMMCs were transfected with plasmids encoding p110γ (WT or KR) with or without plasmids encoding HA-p87 or HA-p101, as well as PH_{Btk}-GFP to monitor PtdIns(3,4,5)P_3 production. Twenty-four hours after transfection, cells were depleted of IL-3 and serum-starved for 3 hours before stimulation with 2 μM adenosine. (**A**) At 0 s (control) and 120 s (adenosine, Ade) after stimulation with adenosine, cells were fixed in 4% *p*-formaldehyde and prepared for microscopy. The localization of PtdIns(3,4,5)P_3 was visualized with PH_{Btk}-GFP. For adenosine-stimulated cells containing p84-p110γ or p101-p110γ complexes, deconvoluted, high-resolution z stacks can be found in movies m1 and m2, respectively. (**B**) p101-p110γ–driven internalization is specific to PtdIns(3,4,5)P_3-binding PH domains. KO BMMCs were reconstituted with p101-p110γ and the indicated fluorescently labeled lipid-binding probes. Stimulation with adenosine (2 μM) was stopped after 15 and 120 s and the localizations of the lipid probes were visualized. Only portions of cells are shown. Left: PH_{Btk}-GFP (Btk) and $PH_{PLCδ}$-RFP (PLCδ) were co-expressed. Middle: Merged picture of PtdIns(3,4,5)P_3-bound (green) and PtdIns(4,5)P_2-bound (red) PH domains after 120 s of stimulation with adenosine. Right: Cells were also transfected with either PH_{Btk}-GFP (Btk) or a lipid-binding defective mutant $PH_{Btk(R28C)}$-GFP (Btk_{R28C}). z stacks were acquired, and images were subsequently deconvoluted.

be assumed that the major output of PI3Kγ in these cells is derived from p101-p110γ complexes. J774 cells could be stimulated with either C5a or C3a to phosphorylate Akt (fig. S8A). C5a and C3a signaling to Akt was PI3Kγ dependent, because the PI3Kγ-specific inhibitor AS252424 efficiently blocked phosphorylation of Akt, whereas TGX-221, which targets PI3Kβ, was ineffective at blocking phosphorylation of Akt (fig. S8A). In J774 cells transfected with a plasmid encoding PH_{Btk}-GFP, stimulation with C5a and C3a led to the association of the PtdIns(3,4,5)P_3 probe with the plasma membrane at early time points (<15 s), but at >45 s, PH_{Btk}-GFP was internalized, as had been observed in adenosine-stimulated KO BMMCs reconstituted with p101-p110γ complexes. Internalization of PtdIns(3,4,5)P_3 thus occurs in cells with endogenous p101-p110γ complexes and might be a pathway alternative to that of the lipid phosphatases to remove PtdIns(3,4,5)P_3 from the plasma membrane (fig. S8B).

DISCUSSION

PI3Kγ is the major PI3K isoform that operates downstream of GPCRs, such as receptors for chemokines, *N*-formyl-Met-Leu-Phe, complement fragments (*16*, *33*, *46*), and murine A3AR (*26*), in hematopoietic cells. GPCRs mediate the activation of PI3Kγ through the release of Gβγ subunits from heterotrimeric G proteins (*18*, *21*, *47*). Biochemical and over-

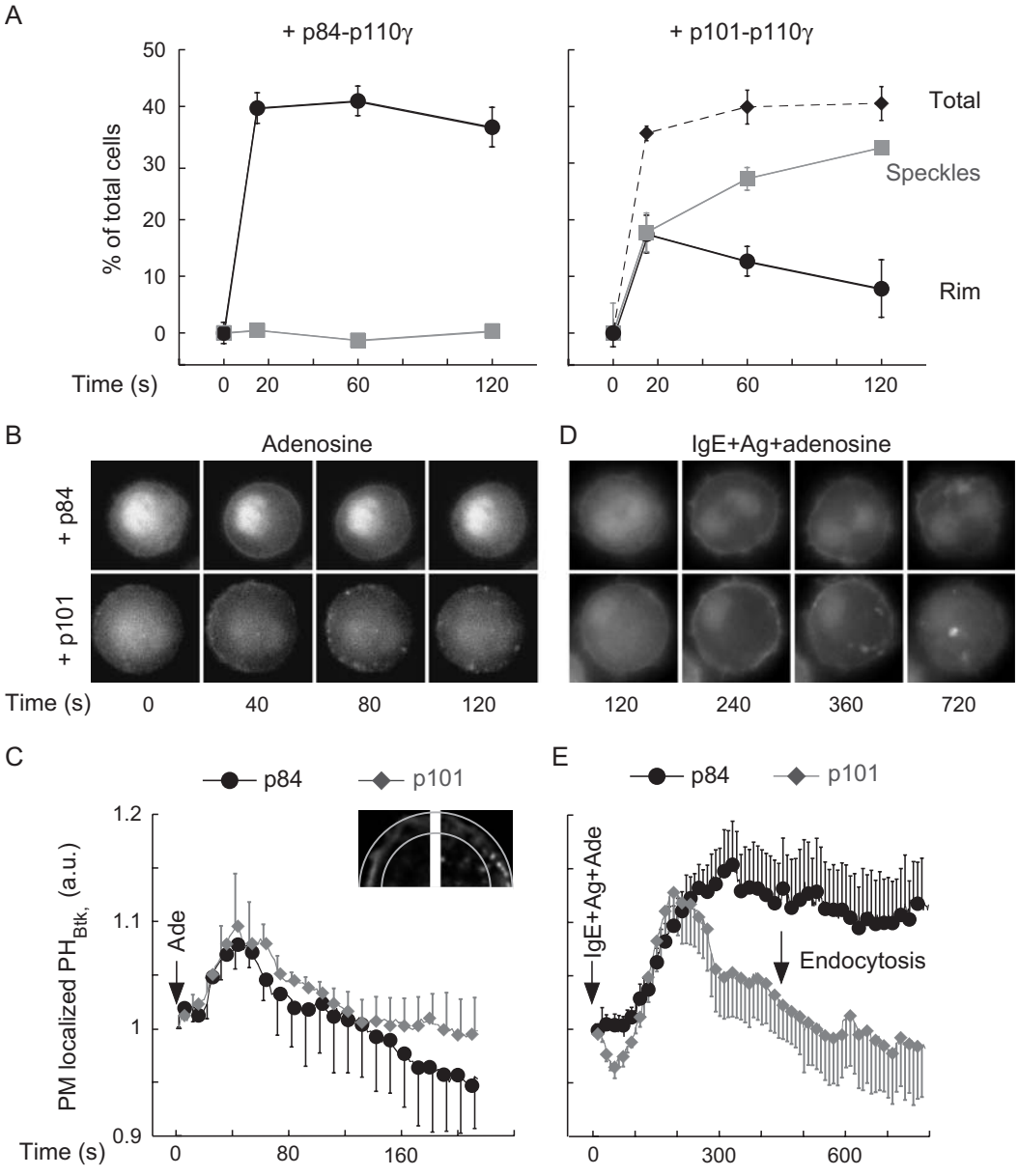

Fig. 6. Time course of the localization of PtdIns(3,4,5)P_3 visualized with PH_{Btk}-GFP after stimulation with adenosine. (**A**) Performed as a population analysis: The localization of the probe was differentiated between membrane staining (rim) and speckles. Quantification of $n = 3$ experiments was performed blindly and the results are expressed as a percentage of the total number of transfected cells. (**B**) Selected time points of live-cell imaging experiments in adenosine-stimulated KO BMMCs reconstituted with p110γ and either p84 or p101 (see also movies m3 and m4). (**C**) Quantification of plasma membrane–localized PH_{Btk}-GFP calculated from live-cell imaging experiments. a.u., arbitrary units, every fifth frame is marked with a symbol. Cells were stimulated with 5 μM adenosine. Data shown are the mean ± SEM for $n = 6$ experiments. Inset is an image that illustrates the region defined as plasma membrane–localized PH_{Btk}-GFP located between the outer circle and the inner circle, for a sample of transfected cells containing p84-p110γ (left) or p101-p110γ (right) complexes. (**D**) Selected time points of live-cell imaging experiments in KO BMMCs reconstituted with p110γ and either p84 or p101. Cells were loaded overnight with anti-DNP IgE (100 ng/ml), washed, and stimulated with DNP-HSA (5 ng/ml; Ag) and 5 μM adenosine for the indicated times. Data shown represent excerpts from movies m5 and m6. (**E**) Quantification of membrane-bound PtdIns(3,4,5)P_3 in IgE+Ag+adenosine–stimulated cells. Data shown are the mean ± SEM of nine experiments for cells containing p84-p110γ complexes, and of seven experiments for cells containing p101-p110γ complexes, and were analyzed as in (C). The low concentration of antigen used here did not trigger translocation of PH_{Btk}-GFP in the absence of adenosine (see fig. S7).

expression studies in nonhematopoietic cells have shown that p101 augments the translocation of PI3Kγ (*41*) and the Gβγ-dependent turnover of PtdIns(4,5)P_2 (*18*, *22*), but the necessity for a PI3Kγ adaptor protein in physiological processes has remained disputed until recently (*24*).

Studies of the expression profiles of PI3Kγ across multiple tissues show that mRNAs encoding p110γ, p84, and p101 are highly expressed in hematopoietic tissues. Whereas the abundance of p101 mRNA was high in tissues and cells that contain lymphocytes [thymus, spleen, and peripheral blood lymphocytes (PBLs)], we could not detect p84 mRNA in PBLs, and the abundance of p84 protein was low in splenocytes. Both adaptor proteins were found in neutrophils and monocytes or macrophages, whereas only p84 was found in mast cells. Overall, p101-p110γ complexes dominate the lymphoid lineage, whereas p84 is found in myeloid cells with varying penetrance. When p110γ was eliminated by gene targeting, p84 protein was destabilized in differentiated cells. Similar reductions were observed for p101 in neutrophils isolated from BM of p110γ null mice (*19*). Because catalytically inactive p110γ (KR) was sufficient to maintain p84 protein in BMMCs, the lipid kinase activity of p110γ is apparently not required for the stabilization of adaptor proteins.

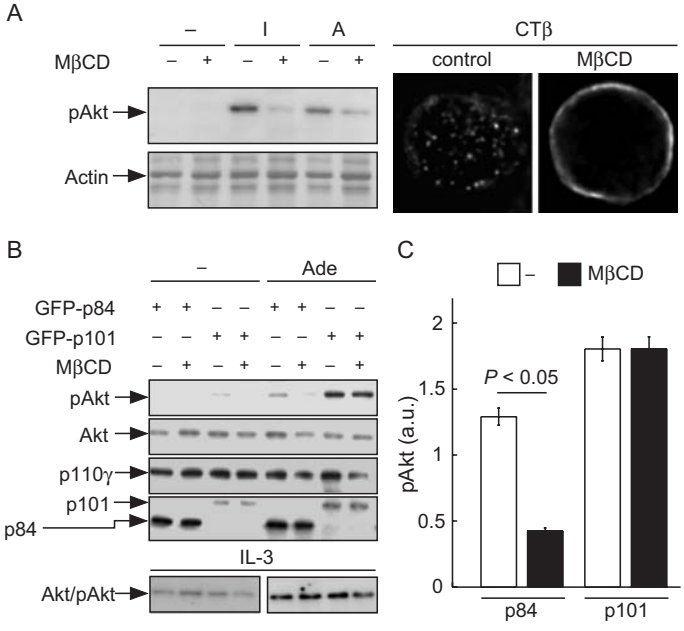

Fig. 7. The function of p84-p110γ, but not p101-p110γ, is sensitive to disruption of lipid rafts. (**A**) Left: WT BMMCs were starved in IL-3–free medium containing 2% FCS for 3 hours with or without 30 min of pretreatment with 5 mM MβCD at 37°C, and then stimulated with 2 μM adenosine or 10 nM IB-MECA for 2 min. Phosphorylation of Akt was determined by Western blotting analysis with an anti-pSer473. Right: Precooled WT BMMCs were incubated at 4°C for 30 min with $Alexa_{555}$-conjugated choleratoxin β-subunit (CTβ) with or without MβCD. Deconvoluted images were acquired after fixation and sedimentation of cells. (**B**) p110γ and GFP fusions of p84 or p101 were expressed in KO BMMCs as indicated (indicators refer to Ade and IL-3 stimulations). Cells were starved as in (A) before phosphorylation of Akt was detected after stimulation with adenosine (Ade) or IL-3 (20 ng/ml for 2 min). One representative experiment of four is shown. (**C**) Quantification of adenosine-triggered phosphorylation of Akt from experiments as shown in (B). Data shown are the mean ± SEM of $n = 4$ experiments.

In mast cells lacking functional p110γ, adenosine was incapable of activating Akt or of triggering the translocation of a probe of PtdIns(3,4,5)P_3 (PH_{Btk}-GFP) to the plasma membrane. In this setting, the combination of adaptor protein with active p110γ was required to restore the responsiveness of KO BMMCs to adenosine. These results show conclusively that p110γ must interact with adaptor protein to generate PtdIns(3,4,5)P_3 downstream of GPCRs.

To date, functional comparisons of p101 and p84 in cells that normally express p110γ have not been performed; in vitro studies have, however, convincingly shown that p101 and p84 activate p110γ by the same basic mechanism (*19*, *22*, *23*). In mast cells, PI3Kγ controls physiological responses in vitro and in vivo (*26*), and both the loss of adaptor protein in p110γ null cells and the inability of monomeric p110γ to rescue GPCR signaling exclude the interference of cross-compensatory effects of the PI3Kγ adaptors in this model.

With the above biochemical studies in mind, it was a surprise that the coexpression of p101 with p110γ did not reconstitute adenosine-dependent degranulation in IgE- and antigen-primed KO BMMCs. The analysis of the abundance of p110γ, p84, and p101 proteins, as well as of their cellular localization, suggested that both types of PI3Kγ complexes were in a position to signal, but that only p84-p110γ did so successfully. In contrast to degranulation, both p84 and p101 could be combined with p110γ to reconstitute adenosine-induced cell motility of KO BMMCs, whereas p110γ alone was insufficient. It is thus evident that GPCRs can link to the chemotactic machinery through both adaptor isoforms. Important roles for p110γ (*16*, *48*) and p101 (*24*) in the chemotaxis of neutrophils have been described previously. That migration of mast cells was proportional to the amount of PI3Kγ complex present strongly suggests that the loss of migratory capacity in p101 null neutrophils (*24*) is due to a reduction in the extent of PI3Kγ signaling rather than to an exclusive link between p101 and cell motility.

The selective promotion of degranulation by p84 suggests that the two adaptor proteins selectively redirect PI3Kγ downstream signaling into separate response patterns. Such selectivity is unlikely achieved through the deployment or interference of different adenosine receptor types, because an A3AR-specific ligand (IB-MECA) and ligands that stimulate all four adenosine receptors (adenosine, NECA) caused phosphorylation of Akt to an equivalent extent in conjunction with both types of PI3Kγ complexes. The observation that PtdIns(3,4,5)P_3 remained at the plasma membrane when it originated from the activity of p84-p110γ, but was endocytosed when its source was the p101-p110γ complex, strongly indicates that the two PI3K complexes produce two distinct PtdIns(3,4,5)P_3 pools at the plasma membrane. That p84-p110γ could signal in lipid microdomains was also supported by its sensitivity to disruption of cholesterol-rich domains by MβCD, whereas adenosine-mediated signaling through p101-p110γ to activation of Akt resisted cholesterol depletion. Cross-linking of FcεRI concentrates the activated receptor in lipid rafts (*43*, *44*), and one could thus speculate that p84-p110γ locally enhances the abundance of PtdIns(3,4,5)P_3 in the vicinity of clustered FcεRI receptors and the associated protein tyrosine kinase cascade. This would imply that degranulation is under the control of a system that integrates the status of the protein tyrosine kinase cascade output with local concentrations of PtdIns(3,4,5)P_3 and relays downstream signals only when the two inputs occur coincidently. Moreover, for efficient degranulation to occur, high threshold concentrations of PtdIns(3,4,5)P_3 have to be reached (*26*), which could be achieved through the selective targeting of p84-p110γ complexes. Such a localized but high-amplitude signal emerging from PI3Kγ could thus overrun the gatekeeper function of the 5′-lipid phosphatase SHIP (*32*, *49*), which continuously attenuates the low-level signal of PtdIns(3,4,5)P_3 produced by p85-associated class IA PI3K under conditions of low concentrations of antigen. If PtdIns(3,4,5)P_3 is localized outside of these areas, as assumed here for p101-p110γ–derived PtdIns(3,4,5)P_3, it has a different route of degradation and can be endocytosed.

Binding of p101 to p110γ has been reported to reduce the basal catalytic activity of PI3Kγ (*50*), and the adaptor proteins can thus be considered as inhibitors and stabilizers of cytosolic PI3Kγ complexes. Here, we showed that these complexes seem to be fully activated only in the correct context and cellular localization. Due to technical limitations, it cannot be concluded presently whether the main action of the adaptors is to restrict access of PI3Kγ to certain sites and responses or to direct PI3Kγ to specific cellular docking sites. The whole process shows similarity to the activation of class IA PI3Ks, in which p85-like regulators block the activity of the catalytic p110 subunit as long as they do not interact with phosphorylated tyrosines on growth factor receptors (*9*, *51*).

The processes in mast cells presented here illustrate that the p84-p110γ and p101-p110γ complexes serve nonredundant but also overlapping functions. In allergy, only the p84-p110γ complex cooperates with IgE- and antigen-dependent signaling in mast cell activation. Our elucidation of adaptor-specific PI3Kγ signaling has a potential therapeutic value, because loss of PI3Kγ function not only attenuates chronic inflammation and allergy, but also modulates cardiovascular parameters (*34*, *52*). Although inhibition of PI3Kγ has been regarded as cardioprotective, such convolution might complicate chronic pharmaceutical targeting of PI3Kγ in inflammatory diseases. Based on our results, tissue selective targeting of PI3Kγ by modulation of the function of adaptor molecules might become possible in the future.

MATERIALS AND METHODS

Cloning of p84, plasmids, and antisera

Murine p84 complementary DNA (cDNA) was obtained from murine 32D cells by reverse transcription PCR (RT-PCR) (for details and primers see Supplementary Materials). Human full-length p84 cDNA was amplified from neutrophils with Pwo polymerase (Roche Diagnostics, Mannheim, Germany) (accession data for p84: *Mus musculus*, AY753194; *Homo sapiens*, AY753192; UniProt: PI3R6_Human; GenBank: *PIK3R6*). The p101 coding sequence (*18*) and p84 were transferred to pcDNA3, pcDNA-HA, and pEGFP vectors for expression. Plasmids for the expression of p110γ expression were previously described (*53*). The PH domain of Btk fused to GFP (pEGFP-PH_{Btk}, donated by T. Balla) was used as a sensor of PtdIns(3,4,5)P_3. The cDNA of the PH domain of PLCδ was subcloned into pTagRFP-N1. Antisera against murine p84 fragment (Met^1 to Glu^{162}) were raised in rabbits and goats and antisera against human p101 fragment (Pro^{575} to Pro^{880}) were raised in rabbits. Protein fragments were expressed as $(His)_6$ fusion proteins from bacterial pQE vectors (Qiagen, Hilden) and purified on Ni^{2+}-NTA beads (Qiagen) according to the manufacturer's instructions.

Cell culture, isolation, and differentiation

To isolate, derive, and culture BMMCs from C57BL/6J mice and mice without functional PI3Kγ [KO_1 (*16*), KO_2 (*33*), and KR (*34*)], cells from fresh BM were resuspended in complete Iscove's modified Dulbecco's medium (IMDM) with 10% heat-inactivated fetal calf serum (HIFCS), 2 mM L-glutamine (Gln), 1% penicillin-streptomycin solution (PEST), 50 μM β-mercaptoethanol (β-ME), and recombinant murine IL-3 (2 ng/ml; Peprotech, Rocky Hill, NJ) and cultured at 37°C and 5% CO_2 for 4 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 second day (*26*). Nonadherent cells were monitored for the presence of FcεRI [with a phycoerythrin (PE)-conjugated hamster antibody to mouse FcεRIα; clone MAR-1, eBioscience, San Diego, CA] and c-kit (rat IgG_{2B} anti-mouse CD117/c-kit; clone 3c1, ImmunoKontact, Bioggo, Switzerland) by fluorescence-activated cell sorting (FACS) analysis. BM-derived macrophages were differentiated in bacterial dishes (Greiner bio-one, Kremsmünster, Austria) at 1×10^6 cells/ml in RPMI 1640 (Sigma) supplemented with HIFCS/Gln/PEST/β-ME and 20% L-929 cell-conditioned medium. Nonadherent cells were collected 5 days later for experiments (*54*). Neutrophils and splenocytes were isolated as described (*16*). The murine macrophage cell line J774 and the rat mast cell line RBL2H3 were cultured in complete DMEM (HIFCS, Gln, PEST), the human mast cell line HMC-1 was cultured in IMDM containing HIFCS/Gln/PEST/ β-ME, and the human monocytic cell line THP-1 was cultured in complete RPMI 1640 (HIFCS/Gln/PEST/β-ME).

Transfections

BMMCs were transfected with the Amaxa Nucleofector according to the manufacturer's protocol (Amaxa). BMMCs (7×10^6 to 10×10^6) were resuspended in 100 μl of nucleofection solution T (Amaxa) to which was added 10 to 15 μg of plasmid DNA (in 15 μl of 10 mM tris and 1 mM EDTA, pH 8.0). Immediately after electroporation, cells were cultured in 5 ml of complete IMDM at 37°C and 5% CO_2 for 5 hours. After a medium change with complete IMDM, transfected cell populations were cultured for 24 hours, after which they were sensitized with mouse anti-DNP IgE (100 ng/ml; mAB SPE-7, Sigma-Aldrich) for degranulation experiments. Stimulations occurred 24 hours after transfection.

Stimulation of BMMCs with adenosine

Twenty-one hours after transfection, BMMCs were collected by centrifugation (160*g* for 3 min), washed, and starved in IL-3–free medium containing 2% fetal calf serum (FCS) for 3 hours (0.5×10^6 to 1×10^6 cells/ml), and were then stimulated with 2 μM adenosine for the indicated time (at 37°C, 5% CO_2). For Western blotting analysis, stimulation of cells was stopped on ice, the cells were collected by centrifugation (16,000*g* for 1 min at 4°C), washed in 1× phosphate-buffered saline (PBS) and lysed at 1×10^7 cells/ml in 2× sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 10% β-ME, 20% glycerol, bromphenol blue]. Proteins were denatured at 95°C for 7 min, and then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting. For microscopy experiments, stimulation was stopped by the addition of an equal volume of ice-cold 10% *p*-formaldehyde in PBS. Wortmannin (500 nM), PTX (100 ng/ml, Sigma), MβCD (5 mM, Sigma), AS252424 (1 μM, Merck-Serono, Geneva), and TGX-221 (1 μM, Cayman Pharma, Neratovice) were added to cells 30 min before stimulation, whereas nocodazole (1 μM, Sigma) was added 3 hours before stimulation.

Western blotting analysis

Proteins were separated by SDS-PAGE and transferred by Western blotting to Immobilon PVDF membranes (Millipore). Mouse monoclonal antibody (mAb) to p110γ (clone H1, Alexis), rabbit mAbs to pSer473-PKB/Akt and to pThr308-PKB/Akt (both from Cell Signaling Technology, Danvers, MA), mouse mAbs to GFP (Roche Diagnostics) and to HA (HA.11, Babco), and the antisera mentioned above were used to detect proteins in the PI3Kγ pathway. Mouse mAb to vinculin was produced from the hybridoma clone VII-F9 (a gift of V. Kotelianski). Secondary antibodies such as horseradish peroxidase (HRP)–conjugated rabbit antibody to mouse IgG and goat antibody to rabbit IgG (Sigma, St. Louis, MO) were visualized by enhanced chemiluminescence (Millipore).

Quantitative Western blotting

After SDS-PAGE, proteins were transferred to Immobilon FL membranes (Millipore) and blocked with Odyssey blocking buffer (LI-COR Biosciences, NE), followed by incubation with primary antibodies in the same buffer. Matched secondary antibodies were goat anti-rabbit IgG-IR Dye 800 (Rockland, Gilbertsville, PA) and goat anti-mouse IgG-Alexa Fluor 680 (Molecular Probes, Eugene, OR). Membrane-bound fluorescence was detected on the Odyssey Infrared Imaging System (LI-COR Biosciences).

Isolation of total RNA, RT-PCR, and qPCR

RNA was isolated from TRIzol-lysed tissues as described by Invitrogen. cDNA was generated with 2 μg 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) was used for qPCR analysis of p84, p110γ, and p101 mRNAs. GAPDH mRNA served as an internal standard to generate calibration curves in which the cycle numbers at the crossing point (CP) were given by

$$\mathrm{CP} = b + a\log([\mathrm{cDNA}])$$

where b was 15.136 and a was –3.2877. Variations in total cDNA content were compensated for with the GAPDH CP values for a given tissue:

$$\mathrm{CP}_{\text{tissue corrected}} = \mathrm{CP}_{\text{tissue}} - (\mathrm{CP}_{\text{GAPDH tissue}} - \mathrm{CP}_{\text{GAPDH external standard}})$$

The relative copy number ($R^{c\#}$) was then calculated as:

$$R^{c\#} = 10^{(4.872 - 0.30354 \times \mathrm{CP}_{\text{sample corrected}})}$$

The efficiency E of PCR was 2.01 or 101%. The above calculation was cross-checked with calibration curves established with linearized plasmids for p110γ and p84. Deviations from the GAPDH-based calculations were in the range of 1.1- to 1.3-fold. cDNA of rat or human origin was amplified by conventional PCR by Taq Polymerase (New England Biolabs, Ipswich, MA) in a T3 Thermocycler (Biometra, Göttingen, Germany). PCR products were separated in 2% agarose gels and visualized with ethidium bromide in a Geneflash imaging system for gel documentation (Syngene, Cambridge, UK). Primers are listed in the Supplementary Materials.

Fluorescence microscopy

After stimulation, cells were fixed by the addition of an equal volume of ice-cold 10% *p*-formaldehyde in PBS. For staining of F-actin, cells were permeabilized in PBS, 1% BSA, 0.1% Triton X-100 and incubated with rhodamine-phalloidin (Molecular Probes) (*55*). Alexa_{555}-conjugated cholera toxin staining (Invitrogen) was performed according to the supplier's instructions. Subsequently, cells were spun onto microscopy slides (at 100*g*) and mounted in Mowiol (Plüss-Stauffer). Images were acquired on an Axiovert 200 M microscope (Zeiss) fitted with a Plan-Achromat 100×/1.4 oil objective and an Orca ER II camera (Hamamatsu) with OpenLab software (Improvision). High-resolution images were generated from 0.2 μM image z stacks, which were then deconvoluted with Volocity 4.0 software.

Live-cell microscopy

Transfected cells (0.5×10^6 to 1.0×10^6/ml, in 0.5 ml) were plated in IMDM containing 2% HIFCS, L-Gln, PEST, 50 μM β-ME on poly-Lys–coated (Sigma-Aldrich) coverslips for 3 hours in live-microscopy chambers (Life Imaging Services, Basel, Switzerland). Live imaging was performed on a Zeiss Axiovert 35 microscope (with a 100× plan-Neofluar 1.30/oil objective), equipped with Micromax heating (to 37°C) and a Princeton Instruments camera system (Trenton, NJ), or on an Axiovert 200 M microscope (Zeiss) fitted with a Plan-Achromat 63×/1.4 oil objective and an Orca ER II camera (Hamamatsu), equipped with a microscope temperature control system (Life Imaging Services). For stimulations, 0.5 ml of modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 20 mM Hepes, pH 7.4) containing 5 μM adenosine or 5 μM adenosine and DNP-HSA [10 ng/ml; where cells were presensitized with anti-DNP IgE (100 ng/ml) overnight] were added carefully to avoid displacement of cells. To monitor the translocation of GFP-PH_{Btk}, images were acquired every 2 s over the indicated time with either Metamorph 4.01 software (Axiovert 35 microscope) or OpenLab software (Axiovert 200 M microscope). Movies were assembled with Volocity 4.0 software. Plasma membrane–localized PH_{Btk}-GFP was determined by measuring the integrated fluorescent intensities of two circular regions of interest (ROIs) with ImageJ software. The first ROI (ROI_{out}) included the whole cell, whereas the second ROI (ROI_{in}), which had a reduced radius, was set such that plasma membrane staining was excluded. The sizes and locations of the ROIs were constant in all frames. Plasma membrane–localized PH_{Btk}-GFP was calculated as $(ROI_{out} - ROI_{in})/ROI_{out}$ for each acquired frame. Division by ROI_{out} in this formula corrects for sample bleaching, because values (in arbitrary units) of all frames were normalized to the first (unstimulated) frame and plotted over time. Intensity surface plots were calculated with ImageJ software.

Single cell–based degranulation assay

BMMCs were incubated in complete IMDM with mouse anti-DNP IgE (100 ng/ml) overnight and then stimulated with DNP-HSA (1 ng/ml; Sigma-Aldrich) with or without 2 μM adenosine for 20 min at 37°C. Cells were then washed in PBS, transferred to annexin V–binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with Cy5-conjugated annexin V (BD Biosciences) for 15 min on ice. FACS analysis was performed on a FACSCalibur with CellQuest software (BD Biosciences). FACS events were gated according to cell size, GFP-positive and GFP-negative cells, and annexin V–Cy5 staining as a measure of degranulation [see Demo *et al*. (*37*) and Martin *et al*. (*56*) for assay validation]. It must be noted that the correlation between annexin V staining and degranulation required validation for each of the stimuli and conditions used (*57*). For experiments with transfected KO BMMCs, degranulation was calculated for GFP-positive and GFP-negative cells separately, as GFP-negative cells define basal degranulation of KO BMMCs. For reconstituted cells, adenosine-induced degranulation was expressed as the difference in the percentage of total cells in M2 (annexin V–positive) after stimulation with IgE and antigen either with (IgE+Ag+Ade) or without (IgE+Ag) adenosine (ΔDegranulation, % of annexin V staining). The borders of the M2 gate were set to discriminate the synergistic action of adenosine from stimulation with IgE and antigen and from apoptotic cells after nucleofection, which were also stained with propidium iodide (PI). See fig. S5 for more information.

Release of β-hexosaminidase

The release of histamine-containing granules was quantified by the determination of β-hexosaminidase in the cell supernatants with a method slightly modified from that of Laffargue *et. al*. (*26*). BMMCs were incubated with anti-DNP IgE (100 ng/ml) overnight and resuspended in modified Tyrode's buffer at 0.5×10^6 to 1.0×10^6 cells/ml at 37°C and 5% CO_2. Degranulation was induced with DNP-HSA (1 ng/ml) with or without 2 μM adenosine for 20 min at 37°C and 5% CO_2. The reaction was stopped and β-hexosaminidase activity was measured with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma). Results are given as the percentage of total Triton X-100–releasable β-hexosaminidase.

Cell migration assays

Migration of transfected BMMCs was assayed in 24-well Transwell Supports (Corning) with 5.0-μm pore polycarbonate membranes for 6 hours. Before the experiment, Transwell membranes were coated with fibronectin (1 μg/ml) in PBS at 4°C overnight, blocked with 4% BSA in PBS for 1 hour at 37°C, and equilibrated in migration medium (IMDM supplemented with L-Gln, PEST, 1% BSA, 50 μM β-ME, and 20 mM Hepes, pH 7.4) for 30 min at 37°C. Transfected BMMCs were washed twice in migration medium.

Cell suspensions (0.5×10^6 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 in the lower well. Fluorescent cells that reached the lower well were quantified with the fluorescent microscope.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/2/74/ra27/DC1
Supplementary Text
Materials and Methods
Fig. S1. Tissue distribution of p110γ and its adaptor subunits (additional tissues).
Fig. S2. p84 is stabilized by p110γ in mast cells.
Fig. S3. Restoration of p110γ does not affect the abundance of endogenous p84 protein.
Fig. S4. Adenosine receptor ligands and PI3Kγ signaling leads to phosphorylation of Akt.
Fig. S5. Illustration of the gating procedures used in single-cell degranulation assays.
Fig. S6. p101-p110γ–dependent endocytosis of PtdIns(3,4,5)P_3 is associated with microtubules.
Fig. S7. Quantification of PH_{Btk}-GFP probes at the plasma membrane after low-level stimulation of BMMCs with IgE and antigen.
Fig. S8. Activation of Akt and localization of PtdIns(3,4,5)P_3 in J774 cells.
Table S1. Sequences of primers used for cloning, antigen production, qPCR, and RT-PCR.
References
Movies m1 to m9

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PUBLICATIONS

Collmann E, Bohnacker T, Marone R, Dawson J, Rehberg M, Krombach F, Owen C, Burkhart C, Hirsch E, Thomas M, Wymann M. PI3K modulates mast cell activation in allergy – all the way from blood to tissue.
Manuscript in preparation

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Clément E, Mesini PJ, Pattus F, Schalk IJ. The binding mechanism of pyoverdin with the outer membrane receptor FpvA in *Pseudomonas aeruginosa* is dependent on its iron-loaded status. Biochemistry. 2004 Jun 22; 43(24): 7954-65

COMMUNICATIONS

March 26-29th, 2008
42nd Annual Scientific Meeting of the European Society for Clinical Investigation, Geneva, SWITZERLAND
Poster title: PI3Kγ in mast cell adhesion, migration and recruitment.

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