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Margarita Dominguez-Villar *Editor*

PI3K and AKT Isoforms in Immunity

Mechanisms and Therapeutic Opportunities

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Margarita Dominguez-Villar
Editor

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Editor
Margarita Dominguez-Villar
Faculty of Medicine
Imperial College London
London, UK

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Abbreviations

IP_3	Inositol 1,4,5-trisphosphate
PDK-1	Phosphoinositide-dependent kinase-1
PI3Ks	Phosphoinositide 3-kinases
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
PtdIns(3,4)P_2	PtdIns-3,4-bisphosphate (PI-3,4-P_2), phosphatidylinositol 3,4-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol-3,4,5-trisphosphate, phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], phosphatidylinositol (3,4,5)-triphosphate ($PI_{3,4,5}P3$), PtdIns-3,4,5-trisphosphate
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate (PIP_2), phosphatidylinositol-(4,5)-biphosphate (PI-(4,5)-P_2), phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP2), phosphorylate phosphatidylinositol-diphosphate (PIP2), phosphatidylinositol 4,5-bisphosphate
PtdIns3P	PtdIns-3-phosphate (PI3P), phosphatidylinositol triphosphate (PIP3), Phosphatidylinositol 3 monophosphate (PtdIns3P), phosphatidylinositol 3-phosphate
PtdIns4P	Phosphatidylinositol 4-phosphate

Introduction to PI3K

Class I PI3K Biology

Tihitina Y. Aytenfisu, Hannah M. Campbell, Mayukh Chakrabarti, L. Mario Amzel, and Sandra B. Gabelli

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Abstract This chapter is an introduction to phosphoinositide 3-kinases (PI3K), with class I PI3Ks as the central focus. First, the various PI3K isoforms in class I are presented with emphasis on their overall structure, subunits, subunit constitutive domains, domain-domain interactions, and functional relevance. This structural analysis is followed by a comprehensive history of seminal investigations into

T. Y. Aytenfisu, H. M. Campbell, M. Chakrabarti: These authors contributed equally.

T. Y. Aytenfisu · H. M. Campbell · M. Chakrabarti · L. M. Amzel · S. B. Gabelli (✉)
Department of Biophysics and Biophysical Chemistry, The Johns Hopkins School of Medicine, Baltimore, MD 21205, USA
e-mail: gabelli@jhmi.edu

S. B. Gabelli
Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

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PI3K activity. Next, we highlight the divergent roles of the isoforms: PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ. This section details signaling pathways in which these PI3K isoforms are involved, including the key upstream regulators of PI3K activity and some downstream cellular effects. Nodes of the PI3K pathway are also presented. Inhibitors of some isoforms are discussed to give an overview of the basis of some immunotherapies that are being used to target cell signaling. Finally, the chapter ends with a discussion of the dysregulation of PI3Ks in diseases including APDS, asthma, arthritis, and oncogenic mutations.

Acronyms

ATP	Adenosine triphosphate
APDS	Activated phosphoinositide 3-kinase δ syndrome
APDS1	Activated phosphoinositide 3-kinase δ syndrome type 1
APDS2	Activated phosphoinositide 3-kinase δ syndrome type 2
ABD	Adaptor-binding domain
BCAP	B-cell associated protein
BCR	B-cell receptor
BLASTp	Basic local alignment search tool for proteins
cSH2	C-terminal Src homology 2
DNA	Deoxyribonucleic acid
DAG	Diacylglycerol
EGFR	EGF receptor
EGF	Epidermal growth factor
FYVE	Fab1, YOTB, Vac1, and EEA1 (the abbreviation from the first four proteins in which the domain was discovered)
FcRs	Fc receptors
GPCRs	G-protein coupled receptors
PIK3CA/B/D/G	Genes encoding p110α/β/δ/γ
GSV	GLUT4 storage vesicles
GMPPNP	Guanosine 5′-[beta,gamma-imido]triphosphate
GLUT-4	Glucose transporter type 4
GSK-3	Glycogen synthase kinase 3
GS	Glycogen synthase
HDX	Hydrogen-deuterium exchange
HD	Helical domain
HDX-MS	Hydrogen-deuterium exchange mass spectroscopy
IGF1R	IGF-1 receptor
ITAM	Immunoreceptor tyrosine-based activation motif
ICOS	Inducible T-cell co-stimulator
IP3	Inositol 1,4,5-trisphosphate
IRS	Insulin receptor substrate
IR	Insulin receptor

IGF-1	Insulin-like growth factor 1
IGF-IR	Insulin-like growth factor 1 receptor
IGFs	Insulin-like growth factors
iSH2	Inter-SH2
IFN-α	Interferon-α
IL2	Interleukin-2
IL2R	Interleukin-2 receptor
IL4	Interleukin-4
IL4R	Interleukin-4 receptor
kDa	Kilodalton
KD	Kinase domain
mABs	Monoclonal antibodies
NGF	Nerve growth factor
nSH2	N-terminal Src homology 2
PDK1	Pyruvate dehydrogenase lipoamide kinase isozyme 1
PDB	Protein data bank
PTEN	Phosphatase and tensin homolog
PtdIns	Phosphatidylinositol
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PIP2	Phosphatidylinositol-4,5-bisphosphate
PI3Ks	Phosphoinositide 3-kinases
pYXXM	Phosphorylated YXXM motif
PDGF	Platelet-derived growth factor
PH	Pleckstrin-homology
PDK1	PtdIns-3,4,5-P3-dependent protein kinase-1
PI-3,4-P2	PtdIns-3,4-bisphosphate
PI3P	PtdIns-3-phosphate
PI4P	PtdIns-4-phosphate
GAP	Rab GTPase activating protein
RGC	Ral-GTPase activating protein heterodimeric complex
RBD	Ras-binding domain
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
mTOR	Mechanistic target of rapamycin
RSV	Rous sarcoma virus
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
TLRs	Toll-like
VEGF	Vascular endothelial growth factor
WNV	West Nile virus

1 Class I PI3Ks at a Glance

Phosphoinositide 3-kinases, PI3Ks, are a family of enzymes that catalyze the phosphorylation of phosphatidylinositol molecules (PtdIns). Class I PI3Ks catalyze the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3-position of the inositol ring to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3; Fig. 1), a signaling lipid that binds to pleckstrin-homology (PH), FYVE, and other lipid-binding domains and regulates cellular functions such as cell differentiation, growth, proliferation, motility, and survival. PI3Ks are heterodimers composed of two chains: a catalytic subunit p110 (having four isoforms: α, β, δ, and γ) and a regulatory subunit, most commonly p85 (also, p50, p55, p101, and p84) named after their apparent molecular weights in SDS-PAGE (Fig. 2a).

Class I PI3Ks are sub-classified into class IA (that includes p110 α, β, or δ) and class IB (comprising only p110γ) based on the sequence of their p110 subunit, the regulatory domain they use, and their preferred substrate. All p110 subunits contain five domains: an N-terminal adaptor-binding domain (ABD), a Ras-binding domain (RBD), a C2 domain that likely binds to the cellular membrane, a helical domain (HD) of unknown function, and a catalytic kinase domain (Fig. 2a) (Walker et al. 1999; Bilanges et al. 2019; Vanhaesebroeck et al. 2012). These p110 subunit domains

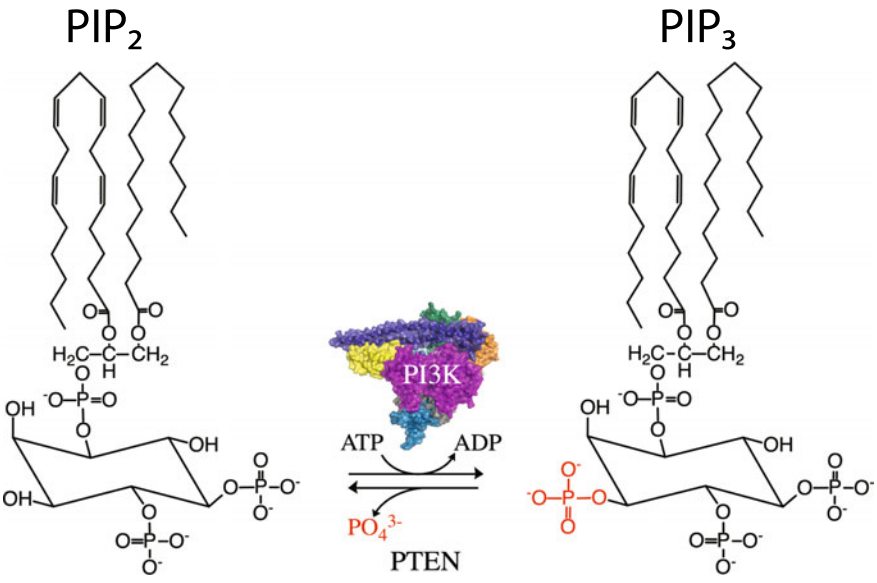


Fig. 1 PIP_3 synthesis by PI3K phosphorylation of PIP_2. PI3Ks catalyze the ATP-dependent phosphorylation of phosphatidylinositol lipids (PtdIns). Class I PI3Ks have substrate specificity for phosphatidylinositol (4,5)-bisphosphate (PIP_2), phosphorylating the molecule at the O3 position to form a phosphatidylinositol (3,4,5)-trisphosphate. PTEN catalyzes the reverse reaction: dephosphorylation of PIP_3 into PIP_2. The newly added and removed phosphate group is shown in red

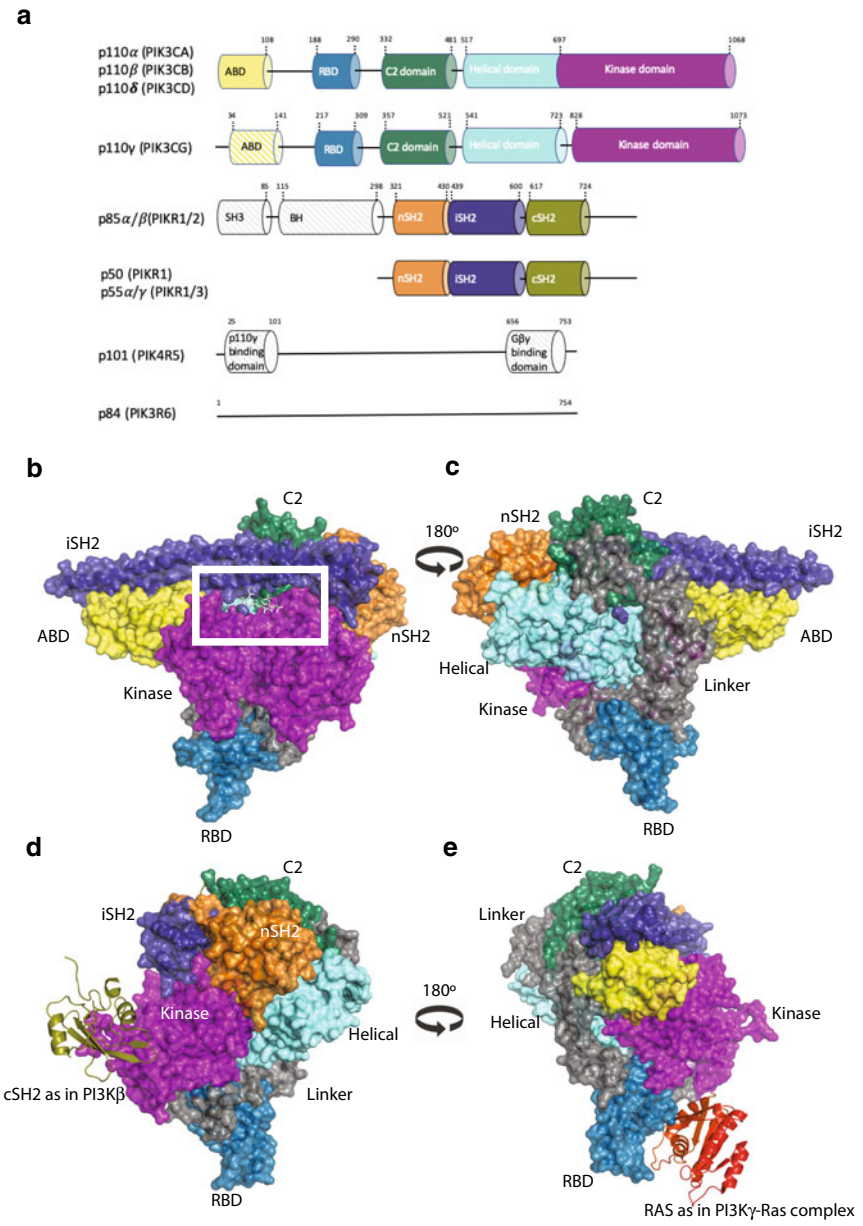
are not arranged side to side like beads-on-a-string. Instead, they are connected by stretches of polypeptide chain, as long as 80 residues, altogether forming a compact structure with dimensions of at least 94 Å × 80 Å × 120 Å (PDB ID 3HHM; Fig. 2b, c). The organization of this structure makes PI3Ks well-suited for robust communication between the domains.

Class IA PI3Ks form heterodimers using one of the following regulatory subunits: p85α, p85β, p50α, p55α, and p55γ; on the other hand, class IB uses either p101 or p84 (also referred to as p87 or p87pikap) (Geering et al. 2007). The class IA regulatory subunit p85 contains five domains: an Src homology 3 (SH3) domain, a RhoGap domain, and two SH2 domains separated by an inter-SH2 (iSH2) coiled-coil region, which binds to the p110 subunit. The regulatory subunits of class IA PI3Ks do not show high sequence homology with those of class IB. The domains of class IB regulatory subunits, p101 and p84, have not been defined yet.

2 Structural Analysis of Class I PI3Ks

The known portion of the PI3Kα structure contains all five p110α domains plus two of the five p85α domains: the nSH2 and the iSH2 (Miled et al. 2007; Huang et al. 2007; Miller et al. 2014). Since this is the isoform for which the reported structure determination includes the largest portion of the molecule, it will be used as the structural paradigm of the class I PI3K isoforms. The p110α subunit contains 1068 residues, and the beginning and the end of each of the five domains (Fig. 2) show the presence of interdomain regions of different lengths, up to 79 amino acids. The adaptor-binding domain, ABD, spanning residues 1–108, has a convoluted fold that includes a doubly twisted β-sheet and an α-helix which spans residues 41–56 (Fig. 3). The ABD makes tight contacts, involving large buried areas, with the kinase domain of p110 and with the iSH2 domain of p85 (Miled et al. 2007; Huang et al. 2007; Miller et al. 2014; Mandelker et al. 2009). The interface of the ABD with the kinase domain of p110 is the locus of several common oncogenic mutations (Gabelli et al. 2010a, b; Huang et al. 2007). The RBD has an α/β fold composed of a 4-stranded antiparallel β-sheet bounded by three α-helices (Fig. 3). The interaction of RBD with Ras has been implicated in some PI3K signaling (Castellano and Downward 2011). The structure of the complex of PI3Kγ with the Ras·GMPPNP complex shows that Ras switch I and switch II regions interact with a loop in the RBD domain of PI3Kγ (Figs. 2 and 3). Mutagenesis experiments show that interactions of the RBD with both regions of Ras are essential for binding PI3Kγ. Besides interacting with the RBD, Ras forms a direct contact with the PI3Kγ catalytic domain. It is proposed that the interactions of PI3Kγ with the two Ras switch regions are likely to be shared by PI3Kα (Pacold et al. 2000).

The C2 domain (residues 330–480) has the typical C2 fold observed in over a hundred of membrane-interacting proteins (Corbalan-Garcia and Gomez-Fernandez 2014; Huang et al. 2007). C2 domains are 150–180 residues long and fold into a β-sandwich formed by two antiparallel sheets, one with four strands and the other


a
p110α (PIK3CA)
p110β (PIK3CB)
p110δ (PIK3CD)
ABD
RBD
C2 domain
Helical domain
Kinase domain
p110γ (PIK3CG)
p85α/β(PIKR1/2)
SH3
BH
nSH2
iSH2
cSH2
p50 (PIKR1)
p55α/γ (PIKR1/3)
p101 (PIK4R5)
p110γ binding domain
Gβγ binding domain
p84 (PIK3R6)
b
C2
iSH2
ABD
nSH2
Kinase
RBD
c
180°
nSH2
C2
iSH2
ABD
Helical
Linker
Kinase
RBD
d
C2
iSH2
nSH2
Kinase
Helical
cSH2 as in PI3Kβ
Linker
RBD
e
180°
C2
Linker
Helical
Kinase
RBD
RAS as in PI3Kγ-Ras complex

◀**Fig. 2** Structural overview of class I PI3Ks. **a** Scheme of the domains of class I PI3K α, β, δ, and γ. The heterodimers of class IA are formed by a catalytic p110α, β, or δ subunit and a p85 regulatory subunit either p85α (or the splicing variants p55 and p50) or p85β. The domains of catalytic subunit p110 include the Adaptor-Binding Domain (ABD, yellow), the Ras-Binding Domain (RBD, marine blue), the C2 domain (forest green), the helical domain (cyan), the catalytic kinase domain (purple). The residue numbers correspond to p110α. The full-length regulatory p85 subunit includes an SH3 domain, a RHO-GAP domain, two Src homology 2 domains called N-terminal SH2 (orange) and C-terminal SH2 (nSH2 and cSH2) and the inter-SH2 domain (iSH2) domain which is a long coiled-coil that mediates binding to the p110 subunit (navy blue). The class IB PI3Kγ catalytic subunit, p110γ, shares four of the C-terminal domains of the class IA isoforms; the p110γ adaptor-binding domain, whose structure is not known, does not have significant homology with those of the class IA isoforms. The regulatory domains of class IB are p101 or p84. **b** Structure of PI3Kα displaying full-length p110α, and the iSH2 and nSH2 domains of p85α; surface representation displays the interaction of the iSH2 of p85α with the ABD of p85α. The binding site of PIP_2 is highlighted with a white rectangle. **c** 180° rotation of the structure from part B, displaying nSH2 (orange) and the long linker domain (gray) connecting the RBD to the C2. **d** 90° rotation of the structure from part B, with PI3Kα (PDB ID 4OVU) structurally aligned to PI3Kβ (PDB ID 2Y3A, mouse) in order to position the cSH2 domain as seen in the PI3Kβ published structure (olive ribbons interacting with kinase domain). **e** Structural alignment of PI3Kα (PDB ID 4OVU) to PI3Kγ in complex with H-RAS (PDB ID 1HE8). H-RAS is shown in red ribbons

with three strands (Fig. 3). As is the case with the C2 domains of other proteins, the one of PI3Kα brings the catalytic (kinase) domain within close proximity of the membrane. It does so in conjunction with the iSH2 domain of p85, with which it has a tight interaction that provides a second anchor between the regulatory domain and the p110. Although the function of many other C2 domains is regulated by Ca^{2+} ion binding, the C2 domain of p110 does not bind Ca^{2+} and also lacks the typical motifs involved in binding the ions (Nalefski and Falke 1996).

The helical domain (HD, residues 517–697) contains ten α-helices. The N-terminal helix (residues 490–505) is almost perpendicular to the remaining nine helices. The HD interacts with the C2 domain and the kinase domain of p110, as well as with the nSH2 domain of p85. These interactions are key for the communication among domains that underlies PI3K class I activation. These HD interfaces are key participants in the activation of PI3Kα, highlighted by the number of "hot-spot" gain-of-function oncogenic mutations that occur at the interface of the HD with the nSH2 in solid tumors (Gabelli et al. 2010b). Notably, in the search for isoform-specific, mutation-specific inhibitors, small molecule fragments (<300 Da) were observed binding to this same interface, suggesting plasticity in these allosteric sites (Miller et al. 2017).

The kinase domain (KD, residues 697–1068) houses the catalytic site of PI3K. The domain has the typical architecture of protein and lipid kinases: two lobes with an α/β fold separated by a large cleft. The N-terminal lobe, the smaller of the two, comprises residues 697–851 and the larger C-terminal domain extends from residue 852 to the last residue, 1068. As in other kinases, catalytic activity requires that two loops from the C-terminal lobe, the activation (933–957) and the catalytic (912–920) loops, adopt the catalytically active conformation. Residues involved in binding the

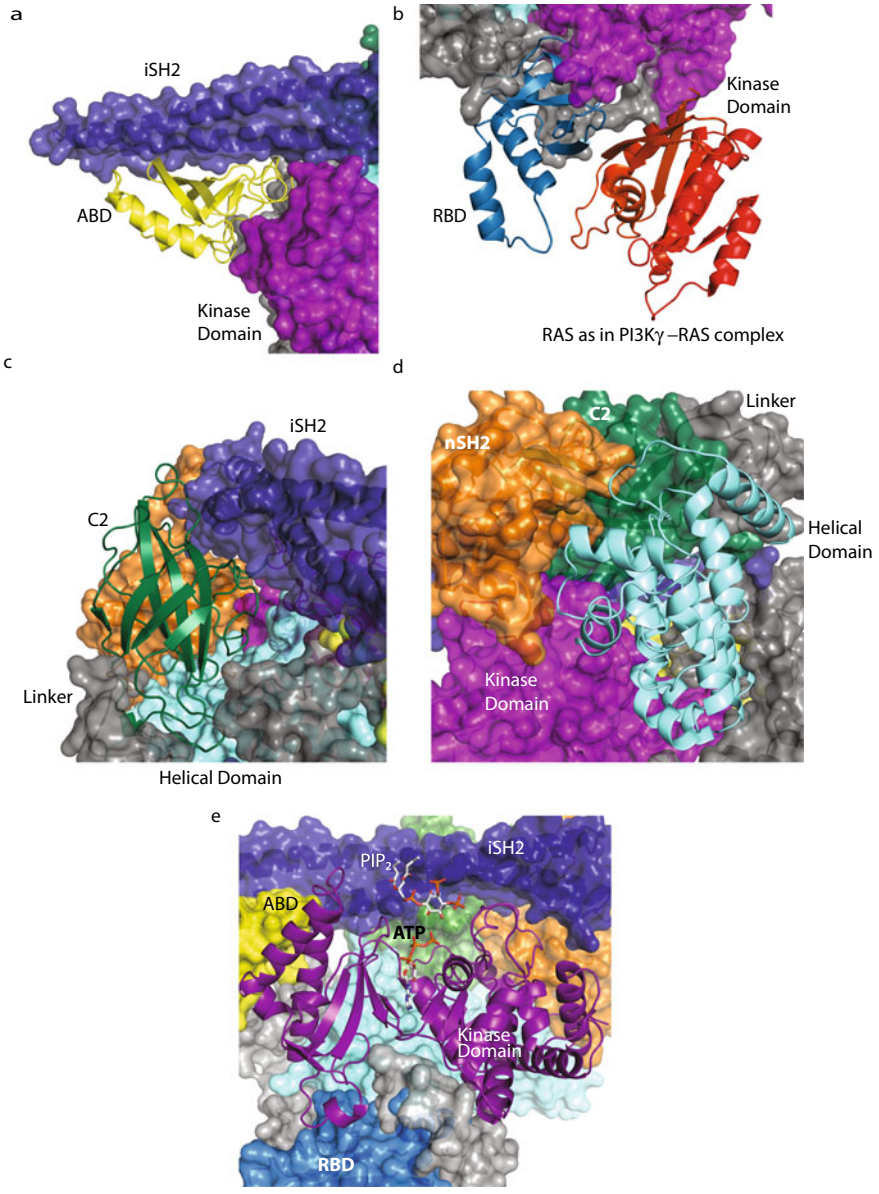


Fig. 3 Domain interactions within p110α. **a** ABD interacts with the iSH2 and kinase domain. **b** RBD (marine blue ribbons) with H-RAS (red ribbons). **c** C2 domain (green ribbons) interacts with nSH2, iSH2, and the helical domain. **d** Helical domain (aquamarine) residues interact with the kinase (purple), nSH2 (orange), and C2 (green) domains. **e** Kinase domain (purple ribbons) with ATP and C6-PIP_2 (white sticks)

two substrates are conserved in the other PI3K class I lipid kinases. The active site opens toward the cell membrane, where PIP_2 is positioned with the inositol phosphate moiety extending out from the membrane and the two fatty acids residing within the phospholipid bilayer. The ATP binding site is deeper in the cleft and further away from the cell membrane. Two residues, one from the catalytic loop (His 917) and one from the activation loop (His 936), are identified as required for catalytic activity (Maheshwari et al. 2017). The KD has extensive contact with the HD involving the N-lobe of the KD, the C-terminal region of the HD, and other regions of the HD with the C-lobe of the KD (Miller et al. 2014).

While the structures of the individual domains of p85 are known, only two of the five p85 domains (nSH2 and iSH2) were included and observed in the most commonly used constructs for structure determination of PI3Kα. The nSH2 domain is a typical Src homology 2 domain consisting of a large β-sheet flanked by two α-helices. This domain is essential for control of the enzymatic activity: it recognizes the phosphorylated tyrosine-containing sequence (Felder et al. 1993; Piccione et al. 1993; Shoelson et al. 1993; Nolte et al. 1996) in the activated receptor tyrosine kinase (RTK) or its phosphorylated substrate, and turns on the PI3K kinase activity. The iSH2 is not an Src homology 2 domain: it is instead a very long coiled-coil that spans the whole length of p110, making contact, especially with the ABD and the C2 domains. It has been shown that some oncogenic mutations affect the interaction of the catalytic subunit with the iSH2 domain, which in turn allows the nSH2 to adopt an activating conformation (Zhao and Vogt 2008b).

The structure of PI3Kβ was determined using all five domains of p110β and the iSH2 and cSH2 of p85β (Zhang et al. 2011). It shows that despite limited sequence identity between p110α and p110β catalytic subunits (<38% as calculated with BLASTp), the structures of the individual domains they have in common, as well as their arrangements, are highly similar. The most distinctive feature of this structure is the inclusion of the cSH2 instead of the nSH2 domain (Fig. 2d). Although it is expected that binding of the phosphorylated tyrosine-containing peptide of RTKs to the cSH2 may affect the activation of the enzyme, its position in the overall structure provides no hint about a possible mechanism.

The structure of PI3Kδ has been determined with p110δ and the iSH2 domain of p85 (Heffron et al. 2016). Initial structures of PI3Kδ were determined with a protein that contained only the last four domains (ΔABDp110δ; RBD, C2, helical, and kinase) (Berndt et al. 2010; Heffron et al. 2011). The three-dimensional arrangement of the heterodimer is similar to the other class IA PI3Ks.

PI3Kγ was the first available structure of the PI3K family; however, 20 years later, the structure of the complex with either of its regulatory subunits still remains to be determined (Walker et al. 2000, 1999). The structure of PI3Kγ was determined with only the four C-terminal domains of p110γ (Walker et al. 1999, 2000). Structural information on p101 and p84 is lacking, as is structural information on the adaptor-binding domain of p110γ, which does not have sequence homology to the one of class IA.

The surfaces of p110γ that interact with p101 have been mapped by hydrogen-deuterium exchange mass spectroscopy (HDX-MS) and by mutational analysis

(Vadas et al. 2013). Both p101 and p84 bind to the RBD, C2, and helical domains of p110γ. The interface of p110γ and p101 has been mapped by HDX (Vadas et al. 2013) and it has been shown that both the catalytic and the regulatory subunits interact with Gβγ. Interestingly, the stoichiometry of the p110γ/p101/Gβγ complex is still under discussion. Two binding sites of Gβγ in the p110γ/p101 complex have been mapped to be $p110\gamma^{RK552DD}$ and $p10^{VVVKR777AAAA}$ (Rynkiewicz et al. 2020; Vadas et al. 2013). On the other hand, only $p110\gamma^{RK552DD}$ has been identified to block activation of the p110γ and p84 complex (Vadas et al. 2013).

3 Historical Overview

3.1 Early Discoveries in Receptor Tyrosine Kinase and Phosphoinositide Signaling

The seminal discoveries of nerve growth factor (NGF) and epidermal growth factor (EGF) by Levi-Montalcini and Cohen in the 1950s and early 1960s (Cohen and Levi-Montalcini 1957, Cohen 1962), and the observation of their effects on neural differentiation and cell proliferation, provided an essential platform for the field of cellular signaling. Early clues to the presence of what we know today as receptor tyrosine kinases (RTKs) can be traced to the 1970s, with the description by Cohen and colleagues of a cellular receptor for epidermal growth factor (EGF) that was later reported to lead to increased levels of phosphorylation in endogenous membrane proteins, as measured by the incorporation of ^{32}P from γ-^{32}P ATP in carcinoma cells upon EGF treatment (Carpenter et al. 1975, 1978). Further insight into the mechanism of activation of such growth factor receptors came with the demonstration that complete bivalent antibodies against the EGF receptor (EGFR), but not monovalent Fab fragments, could induce signaling responses leading to DNA synthesis (Schreiber et al. 1983). This finding followed an earlier report in 1978 that demonstrated a similar phenomenon in which bivalent antibodies to the insulin receptor, and not monomeric Fab fragments, could activate signaling (Kahn et al. 1978) These observations suggested not only that receptor dimerization or clustering was a necessary step in the signaling process, but also that growth factor receptors functioned through a signal transduction mechanism that did not involve ligand internalization, as had been hypothesized in the field (Vigneri et al. 1978; Podlecki et al. 1987).

In 1979, Hunter and colleagues reported what at the time was an unusual observation of tyrosine residue phosphorylation following the immunoprecipitation of murine cells infected with oncogenic polyoma virus (Eckhart et al. 1979) which was followed by a similar observation associated with immunoprecipitation assays of oncogenic Rous Sarcoma virus (RSV) (Hunter and Sefton 1980). The early 1980's saw a flurry of activity that conclusively established, for the first time, signaling by tyrosine phosphorylation associated with the binding of growth factors, including EGF, platelet-derived growth factor (PDGF), and insulin, to their cognate receptors

(Ushiro and Cohen 1980; Kasuga et al. 1982; Ek et al. 1982; Hunter and Cooper 1981; Cooper et al. 1982). The advent of improved cDNA cloning techniques led to the elucidation of the full amino acid sequence and detailed molecular characterization of several receptors, including EGFR, the insulin receptor, insulin-like growth factor (IGF-1) receptor, and PDGF receptor, and to the recognition that these receptors had shared domain architecture (Ullrich et al. 1985, 1986; Ebina et al. 1985; Yarden et al. 1986). This fact was creatively exploited by Ullrich and colleagues, who designed a chimeric receptor consisting of the extracellular domain of the insulin receptor fused to the transmembrane and intracellular domains of EGFR and demonstrated the induction of tyrosine kinase activity in the EGFR cytoplasmic domain by insulin binding (Riedel et al. 1986).

During this time, a connection between RTKs and phosphoinositide signaling was also becoming clearer. Early clues came from several papers in the mid-1980s that reported the presence of phosphatidylinositol (PtdIns) kinase activity associated with oncoproteins of polyoma virus, RSV, and the avian sarcoma virus UR2 (Sugimoto et al. 1984; Macara et al. 1984; Whitman et al. 1985; Kaplan et al. 1986). At the time, the field was focused on elucidating the mechanism of PtdIns processing and the fate of PtdIns-4,5-bisphosphate (PIP_2) upon receptor activation, specifically involving the phospholipase C pathway that led to the production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Akhtar and Abdel-Latif 1980; Michell et al. 1981; Berridge 1983). Seminal papers by Whitman et al. and Traynor-Kaplan et al. in 1988 led to a paradigm shift: their reports identified two novels PtdIns species, PtdIns-3-phosphate (PI3P) and PtdIns-3,4,5-trisphosphate (PIP_3) (Whitman et al. 1988; Traynor-Kaplan et al. 1988). The presence of these phosphatidylinositol species represented the first reported instance of activity that we now ascribe to PI3K: phosphorylation at the 3'-OH group of an inositol lipid, i.e., that of a phosphoinositide-3-kinase.[1] In the following year, Cantley and colleagues further demonstrated the presence of 3′-PtdIns species arising from PtdIns, PtdIns-4-phosphate (PI4P), and PtdIns-4,5-bisphosphate in anti-phospho-tyrosine immunoprecipitated cells and smooth muscle cells stimulated with PDGF (Auger et al. 1989), with PtdIns-3,4-bisphosphate (PI-3,4-P_2) reported as another novel PtdIns species. Phosphoinositide 3-kinase activity, resulting in the same species (PI3P, PI-3,4-P_2, and PIP_3), was also found to be stimulated upon binding of insulin to insulin receptors in cells immunoprecipitated with anti-phospho-tyrosine and insulin receptor antibodies (Ruderman et al. 1990). However, a more discerning characterization of these cellular phosphoinositide led to observations that suggested, although not realized at the time, that PI3-Kinase activity may not be represented by a single signaling pathway. For instance, it was reported that while cells transformed with polyoma virus could still be stimulated by the addition of the growth factor PDGF, the phosphoinositide species PI3P did not accumulate beyond a certain elevated level and that PI3P levels in a neuroblastoma cell line remained unchanged relative to control cells even after stimulation by the known RTK growth factor IGF-1 (Ulug et al. 1990;

[1] Some papers refer to phosphoinositide-3-kinases as phosphoinositol-3-kinases or PtdIns-3-Kinases.

Poyner et al. 1990). Furthermore, while PI3P could be found in transformed and non-transformed cells both in their growth phase and when confluent, PtdIns-3,4-bisphosphate and PIP_3 were uniquely detected in cells that were transformed, in the growth phase, or stimulated by PDGF (Serunian et al. 1990).

As we know today, PI3P is the product of class II and class III PI3Ks, whose signaling pathways diverge from class I PI3Ks. Moreover, it was established that PtdIns-3,4,5-trisphosphate (PIP_3) was a direct product of PI-4,5-bisphosphate (PIP_2) and that the local concentration of the trisphosphate species increased rapidly following agonist-mediated receptor stimulation, demonstrating that PIP_3 could be the primary output of parallel signaling pathways in RTKs and G-protein coupled receptors, and leading to speculation that it might serve as an important second messenger in cells (Stephens et al. 1991; Hawkins et al. 1992). This analysis also postulated that the cellular pool of PI-4,5-bisphosphate was a direct product of 5'-Kinase activity on the precursor PI4P, itself arising from PtdIns (Stephens et al. 1991).

3.2 Phosphoinositide Kinases: Toward a Molecular Understanding of PI3K Activity

Despite the identification of activity ascribed to a Phosphoinositide-3-Kinase and detailed analysis of cellular phosphoinositide pools and interconversion pathways, a molecular characterization of PI3K and the mechanism by which it was activated by RTKs remained largely unclear until the early 1990s. Initial clues were provided by several reports of the presence of an 85 kDa tyrosine-phosphorylated protein that appeared concomitantly with phosphoinositide kinase activity when cells were stimulated by PDGF or transformed by oncoproteins, suggesting that this phosphoprotein may in fact be a substrate of the cognate receptors for such factors (Kaplan et al. 1987; Courtneidge and Heber 1987; Kazlauskas and Cooper 1989; Escobedo et al. 1991a). An important development was made by Cantley and colleagues, who demonstrated that the protein exhibiting Phosphoinositide-3-Kinase activity co-eluted as a heterodimer of the previously described 85 kDa protein and a previously unreported doublet of a 110 kDa protein, yielding an apparent mass of 190 kDa (Carpenter et al. 1990). Intriguingly, their report identified two slightly different 110 kDa proteins, suggesting that there may be multiple isoforms present. However, it was not known at that time whether it was the 85 kDa protein or the newly discovered 110 kDa proteins in the complex that conferred PI3K activity. An answer to this question came from several studies that utilized cDNA cloning techniques to demonstrate that the 85 kDa protein, now known as p85α, contained an N-terminal Src homology (SH) 3 and two SH2 domains, and did not intrinsically possess Phosphoinositide-3-Kinase activity (Escobedo et al. 1991b; Skolnik et al. 1991; Otsu et al. 1991).

A second p85 isoform, termed p85β, was also identified at this time (Otsu et al. 1991). The SH2 domain had been originally recognized as a 100 amino acid sequence present in oncoproteins, N-terminal to the catalytic domain of cytoplasmic (i.e., non-receptor) tyrosine kinases (Sadowski et al. 1986), and was later found to be a common feature of proteins that bound to phosphotyrosine-containing sequences of activated growth factor receptors (Anderson et al. 1990; Matsuda et al. 1990; Moran et al. 1990). This was also found to be true for the SH2 domains of p85 (Hu et al. 1992; Klippel et al. 1992; McGlade et al. 1992; Yamamoto et al. 1992). These findings suggested that the 110 kDa protein (p110) may in fact contain the catalytic activity of PI3K (Shibasaki et al. 1991). This hypothesis was confirmed in 1992 by Hiles et al., who demonstrated through cDNA cloning that p110 was sufficient for phosphoinositide-3-Kinase activity and formed a stable complex with p85α (Hiles et al. 1992a). While this analysis did not yield any insight into sequence motifs in p110 that could explain phosphoinositide activity, it was found that p110 contained homology to the *Saccharomyces cerevisiae* Vps34p protein, involved in protein targeting to the yeast vacuole and in vesicular transport, leading to the intriguing possibility that Vps34p was a yeast phosphoinositide-3-Kinase and the postulate that p110 may have similar endosomal transport roles (Hiles et al. 1992b). It was later found that Vps34p produces PI3P from PtdIns, functions in complex with a secondary protein, Vps15, to mediate endosomal protein sorting, and is conserved between yeast and mammalian cells (Schu et al. 1993; Stack et al. 1993). It was beginning to be recognized that phosphoinositide-3-Kinase activity on PtdIns resulting in PI3P differed from the activity on PtdIns-4,5-bisphosphate, resulting in PIP_3 and the degradation product PtdIns-3,4-bisphosphate, and likely represented a distinct enzymatic process (Stephens et al. 1994b).

A novel p110 isoform, termed p110β, was also characterized at this time, confirming earlier results that had suggested the presence of multiple p110 isoforms (Hu et al. 1993) (Fig. 2). In the following years, work focused on the identification of additional PI3K genes led to the discovery of p110γ, which was found to be a downstream effector of G-proteins (Stephens et al. 1994a; Stoyanov et al. 1995a) and p110δ (Vanhaesebroeck et al. 1997; Chantry et al. 1997) (Fig. 2). Coupled to the discovery of p110γ was the identification of a new regulatory subunit different from p85: a 101 kDa protein, termed p101, was found to associate with Gβγ subunits, activate p110γ by more than 100-fold, and associate with p110γ in a 1:1 complex (Stephens et al. 1997). Also discovered by PCR amplification of conserved peptide sequences in lipid kinases were proteins containing a C-terminal C2 domain, representing a distinct class of phosphoinositide-3-Kinase (Virbasius et al. 1996; Chantry et al. 1997; Arcaro et al. 1998; Ono et al. 1998; Misawa et al. 1998). These findings led to the formal classification of phosphoinositide-3-Kinase into the three classes as we know them today: class I PI3-Kinases, the focus of this chapter, consisting of proteins with a p110 catalytic subunit, class II PI3-Kinases, consisting of proteins having a C-terminal C2 domain, and the class III PI-3-Kinase family containing Vps34 as a unique member (Zvelebil et al. 1996; Vanhaesebroeck et al. 1997; Domin and Waterfield 1997). Concomitant and central to all these discoveries was the use

of wortmannin (Arcaro and Wymann 1993) and LY294002 inhibitors (Vlahos et al. 1994) that helped position PI3Ks as a possible target.

4 Phosphoinositide 3-Kinase Signaling

Despite the elucidation of multiple classes of phosphoinositide-3-Kinases (PI3-Kinase), and the molecular characterization of the catalytic and regulatory domains of class I PI3Ks, the downstream effectors involved in cellular responses associated with PI3K signaling remained largely uncharacterized until the late 1990s. AKT (alternatively referred to as Protein Kinase B or RACα), which had been found to contain a pleckstrin-homology (PH) domain (Haslam et al. 1993; Mayer et al. 1993), a 100 amino-acid sequence capable of binding PtdIns-4,5-bisphosphate (Harlan et al. 1994; Garcia et al. 1995; Lemmon et al. 1995), was identified to be a downstream effector of PI3-Kinase signaling (Alessi et al. 1996; Burgering and Coffer 1995; Kohn et al. 1995). An initial postulate that the PH domain of AKT associated with 3'-phosphoinositides (Franke et al. 1995) was verified by subsequent work that established the association of the AKT PH domain with PtdIns-3,4-bisphosphate and PIP_3 (Franke 1997; Frech et al. 1997; Klippel et al. 1997). However, the mechanism of AKT activation remained unclear until an upstream kinase, itself containing a PH domain, was discovered and termed PtdIns-3,4,5-P3-dependent protein kinase-1 (PDK1), providing a partial explanation for PI3K-mediated AKT signaling (Alessi et al. 1996, 1997a, b; Stokoe et al. 1997). The secondary factor involved in AKT activation, now known to be the Rictor/mTOR complex, was not identified until 2005 (Sarbassov et al. 2005). Today, AKT is known to have more than 100 downstream signaling substrates (Manning and Cantley 2007; Manning and Toker 2017) underlying its central importance in mediating cellular responses to extracellular stimuli. Two of these include Glycogen synthase kinase 3 (GSK-3) (Embi et al. 2005) and Glucose transporter type 4 (GLUT-4) (Fukumoto et al. 1989), both implicated in AKT-mediated insulin signaling (Cross et al. 1995; Hurel et al. 1996; Hill et al. 1999; Kupriyanova and Kandror 1999), and discussed in the following subsection.

4.1 Class I PI3K Signaling Downstream of RTKs

In humans, there are 58 RTKs grouped into 19 subfamilies (Robinson et al. 2000; Trenker and Jura 2020). These RTKs are activated by their physiological ligands, which include growth factors and hormones. Activation results in oligomerization (Schlessinger 1988) and tyrosine autophosphorylation *in trans,* often due to the abrogation of autoinhibitory interactions in *cis* (Hubbard et al. 1998; Mohammadi et al. 1996; Lemmon and Schlessinger 2010). Class I PI3Ks associate with RTKs (Backer et al. 1992b; Bjorge et al. 1990; Coughlin et al. 1989; Guo et al. 1995; Varticovski et al. 1989) and are recruited via binding of the SH2 domains of class I adaptor

proteins to phosphorylated tyrosine residues on activated RTKs and RTK substrates (Inukai et al. 2001; Kavanaugh et al. 1992; Levy-Toledano et al. 1994; Ueki et al. 2000). These SH2 domains have high affinity and specificity for the phosphotyrosine motif Y-M/V-X-M (Felder et al. 1993; Piccione et al. 1993; Shoelson et al. 1993; Nolte et al. 1996). The class I PI3Ks are subdivided into two classes, class IA and class IB, based on adaptor subunit association. Only the class IA isoforms PI3Kα, PI3Kβ, and PI3Kδ, which associate with adaptor proteins containing two SH2 domains, signal downstream of RTKs, although PI3Kβ can also be activated by G-protein coupled receptors (GPCRs) (Kurosu et al. 1997). There are seven class I PI3K adaptor proteins (p85α, p85β, p55α, p55γ, p50α, p101, p84) of which five associate with class IA isoforms: p85α, p85β (Otsu et al. 1991) p85α splice variant p55α (Antonetti et al. 1996; Inukai et al. 1996), p55γ (Pons et al. 1995) and p85α splice variant p50α (Inukai et al. 1997).

Of the many ligands that bind and activate RTKs, the peptide hormone insulin and the closely related insulin-like growth factors (IGFs), IGF-I and IGF-II, have a highly consequential physiological role. These ligands collectively regulate cellular pathways influencing cell survival, growth, lipid, protein and glycogen synthesis, and glucose transport (Boucher et al. 2014; Liefers-Visser et al. 2017; Malaguarnera and Belfiore 2011) (Fig. 4). Substrates of the insulin and IGF-I receptors (IR and IGF1R, respectively) include the Insulin Receptor Substrate proteins (IRS, IRS-1 through IRS-6), although the primary mediators of signaling are IRS-1 (White et al. 1985; Sun et al. 1991) and IRS-2 (Sun et al. 1995). IRS-3 is not expressed in humans (Björnholm et al. 2002). IRS-4 has limited tissue expression (Björnholm et al. 2002; Uchida et al. 2000) and neither IRS-5 nor IRS-6 associates with PI3K p85 (Cai et al. 2003).

Both IRS-1 and IRS-2 associate with the p85 adaptor subunit of PI3K (Altschuler et al. 1994; Folli et al. 1992; Hers 2007; Myers et al. 1992; Yonezawa et al. 1992; Inoue et al. 1998; Valverde et al. 1998) leading to PI3K activation and the stimulation of downstream effectors (Fig. 4). However, PI3K activation can also be mediated by direct p85 regulatory subunit association with activated insulin receptors (Valverde et al. 1998; Ottinger et al. 1995; Van Horn et al. 1994; Staubs et al. 1994). Activated class I PI3Ks catalyze the phosphorylation of the 3'-OH group on the inositol ring of PtdIns-4,5-P_2, leading to the production of PIP_3, which acts as a scaffold for proteins containing PH domains (Isakoff et al. 1998; Kavran et al. 1998; Lietzke et al. 2000; Rameh et al. 1997), notably AKT (Gray et al. 1999; James et al. 1996; Thomas et al. 2002) and PDK1 (Currie et al. 1999; Stephens et al. 1998; Komander et al. 2004). PDK1 has been shown to associate with the cell membrane in response to insulin and growth factor stimulation, facilitating interactions with AKT (Anderson et al. 1998; Filippa et al. 2000). AKT is maximally activated when phosphorylated on both the conserved threonine residue Thr 308 (via phosphorylation by PDK1) and Ser 473 (by the Rictor/mTOR complex) (Hresko and Mueckler 2005; Scheid et al. 2002), or on Thr 308 concurrently with Ser 477 and Thr 479 (Chu et al. 2018). PIP_3 levels are regulated by Phosphatase and Tensin homolog (PTEN), a protein associated with tumor suppressor activity, which dephosphorylates the 3′ position

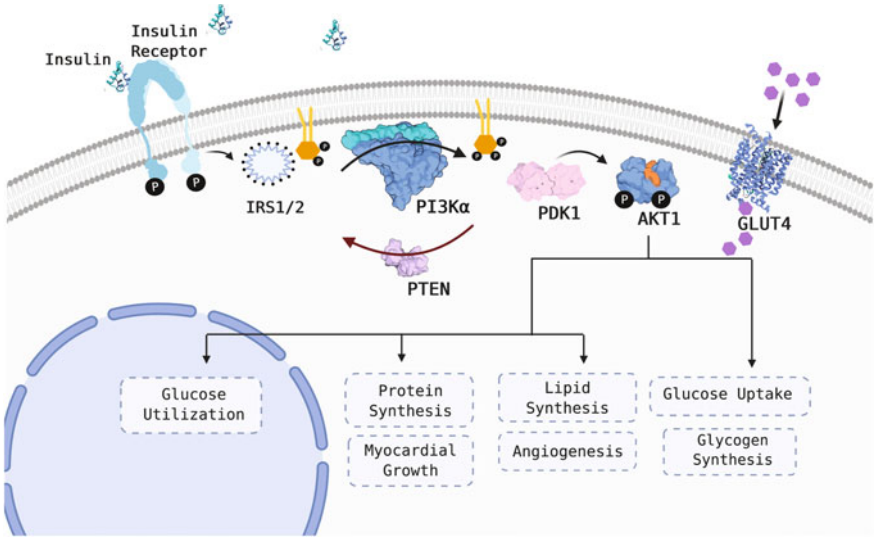


Fig. 4 Receptor tyrosine kinase signaling by PI3Kα. PI3Kα signals downstream of several RTK including sIR, IRL, PDGF (Tsolakos et al. 2018), VEGF, EGFR (Juvin et al. 2013), etc. Binding of insulin to the dimeric insulin receptor activates its tyrosine kinase and causes a transphosphorylation of its cytoplasmic domains. The activated insulin receptor tyrosine kinase domains are recognized by adaptor proteins such as the insulin receptor substrate (IRS 1/2). Phosphorylated IRS-1 is sensed by PI3Kα through binding at the nSH2 domain of the regulatory subunit of PI3Kα (p85α). Subsequently, activated PI3Kα phosphorylates PIP_2 at the 3-position hydroxyl to synthesize PIP_3. PTEN, a phosphatase, and onco-suppressor dephosphorylates PIP_3 to PIP_2 to maintain the balance of the phosphoinositides. PDK1, after being recruited to the membrane by PIP_3, phosphorylates AKT1 which is also been recruited to the membrane by PIP_3. AKT in its phosphorylated state has many targets within the cell, some of which cause the following cellular functions: glucose uptake mediated by GLUT4 (PDB ID 4ZW9), glycogen synthesis, lipid synthesis, angiogenesis, protein synthesis, myocardial growth, and glucose utilization. This rendition was created in BioRender using built-in icons or generating icons from the deposited structures of insulin (PDB ID 1TRZ), insulin receptor kinase (built-in icon from BioRender), PI3Kα (PDB ID 4OVU), AKT1 (PDB ID 6NPZ), PTEN (PDB ID 1D5R), and PDK1 (PDB ID 1HW1). The structure of GLUT3 (PDB ID 4ZW9) was used for GLUT4 since they are of sufficient homology at this resolution

of PIP_3 to regenerate PtdIns-4,5-P_2 (Maehama and Dixon 1998) and functions as a regulator of PI3K signaling (Nakashima et al. 2000; Sun et al. 1999).

Common examples of PI3K signaling include pathways mediated by insulin and its downstream effectors such as Glycogen Synthase Kinase 3 (GSK3). GSK3, a protein with two identified isoforms (Woodgett 1990), is one of the direct downstream substrates of AKT (van Weeren et al. 1998) and participates downstream of multiple signaling pathways (Ding et al. 2000; Fang et al. 2000). GSK3 preferentially phosphorylates substrates that have already been phosphorylated and recognize the sequence motif SXXXpS, where pS is a phosphoserine residue (Fiol et al. 1990; Wang et al. 1994). In addition to AKT, other protein kinases have also been implicated in GSK3 inactivation by phosphorylating either Ser 21 in GSK3α or Ser 9 in

GSK3β in response to insulin (Sutherland and Cohen 1994; Sutherland et al. 1993). However, GSK3 inhibition by AKT in response to insulin has been shown to be independent of the insulin or IGF1-stimulated inhibition of GSK3 involving these other kinases (Cross et al. 1997; Moule et al. 1995; Shaw and Cohen 1999).

Structural studies have suggested a mechanism by which AKT and other proteins are able to inactivate GSK3 (Dajani et al. 2001; Frame et al. 2001; Ter Haar et al. 2001). The phosphoserine in SXXXpS motifs of GSK3 substrates interacts with a positively-charged phosphate-binding pocket that properly orients the catalytic serine residue, which is positioned four residues upstream of the pS phosphoserine. Phosphorylation of the Ser 9 or Ser 21 position of GSK3 by AKT thus results in a "pseudo-substrate" that inhibits kinase activity by preventing the binding of other pre-phosphorylated GSK3 substrates. One of the direct substrates of GSK3 is glycogen synthase (GS), an enzyme that is involved in glycogen synthesis and is phosphorylated by GSK3 on residues Ser 641, Ser 645, Ser 649, and Ser 653 (Poulter et al. 1988; Rylatt et al. 2005). The phosphorylation of Ser 641 and Ser 645 in GS by GSK3 appears to result in significant loss of enzyme activity (Skurat and Roach 1995; Wang and Roach 1993). GS activity is increased in response to signaling by insulin due to a reduction in phosphorylation of the residues that are phosphorylated by GSK3 (Lawrence and Zhang 1994; Lawrence et al. 1983; Parker et al. 2005).

Insulin-mediated signaling through PI3K and AKT (specifically the AKT2/PKBβ isoform) also appears to regulate the complementary activity of Glucose transporter type 4 (GLUT-4) (Bae et al. 2003). GLUT4 is a glucose transporter that in the absence of insulin is found in intracellular vesicles. In adipocytes and skeletal muscle, the GLUT4 storage vesicles (GSV) are translocated to the plasma membrane in an insulin-dependent manner (James et al. 1988, 1989) (Fig. 4). Downstream targets of AKT2 that are posited to influence GLUT4 translocation include the Ral-GTPase activating protein heterodimeric complex (RGC) and the Rab GTPase activating protein (GAP) TBC1D4/AS160 (Chen et al. 2011, 2014; Sano et al. 2003; Zeigerer et al. 2004). The eventual activation of the protein RalA is implicated in the regulation of GLUT4 translocation, and RGC involvement in this process has been demonstrated through direct AKT2 phosphorylation of the RGC β-subunit or through interactions of the RGC α-subunit with 14-3-3 proteins. Other pathways leading to RalA activation have been found to be mediated through Rac1 and the Rab protein Rab10 (Karunanithi et al. 2014; Takenaka et al. 2015). The Rab GAP TBC1D4/AS160 has been found to regulate several Rab G-proteins, many of which have been found to associate with vesicles containing GLUT4 (Larance et al. 2005; Mîinea et al. 2005), and have been also shown to interact with 14-3-3 proteins (Ramm et al. 2006). Thus, the phosphorylation of TBC1D4/AS160 by AKT2 abrogates its GAP activity, leading to an increase in the GTP-loaded form of AS160 substrates and upregulation of GLUT4 vesicle translocation. Recent advances in cellular imaging have permitted visualization of the dynamic GLUT4 trafficking between intracellular compartments and the plasma membrane in response to insulin (Fujita et al. 2010; Lizunov et al. 2005; Xu et al. 2011).

5 Divergent Roles of Class I Isoforms

All class I PI3K isoforms catalyze the same chemical reaction and appear to exhibit significant structural homology. The main distinguishing features between the isoforms are found in their upstream regulators, their relative expression in different cells, and downstream functions. In this section, we focus on these distinguishing features among all class I isoforms: PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ.

5.1 PI3Kα

PI3Ks have been shown to link growth factors to signaling downstream of RTKs. Specifically, PI3Kα is recruited by activated insulin receptor (IR) (Backer et al. 1992a), the insulin-like growth factor receptor (IGF-IR), HER2/ErbB-2, hepatocyte growth factor receptor (Bardelli et al. 1992), c-kit (Nolte et al. 1996), and PDGF (Escobedo et al. 1991a) (Fig. 4). Vascular Endothelial Growth Factor (VEGF) has been also shown to activate p110α in endothelial cells with surprising isoform selectivity (Graupera et al. 2008). Moreover, PI3Kα is essential in signaling for vascular development. The inactivation of p110α either ubiquitously or specifically in endothelial cells was shown to lead to embryonic death as a result of severe deficiencies in angiogenesis and vascular remodeling. Growth factors, such as the insulin growth factor, signal downstream of PI3Kα to promote the adaptive physiological heart growth response to exercise which antagonizes pathological growth (Shioi et al. 2003; Vega et al. 2017). Furthermore, treatment with PI3Kα inhibitors obliterates the favorable effects of exercise. PI3Kα can protect from cardiac hypertrophy in diabetic cardiomyopathy, which is associated with fibrosis and dysfunction, as well as pressure overload in myocardial infarction (McMullen et al. 2003). Boosting PI3Kα signaling is a beneficial intervention in diabetes-induced cardiac injury (Li et al. 2017) and can rescue cardiac disfunction (Weeks et al. 2012).

Although PI3Kα has a Ras-binding domain, which is common to all class I PI3Ks, its relevance and order of activation is still a matter of active investigation. Activation of PI3Kα by Ras can be inhibited by the p85 regulatory subunit. However, this inhibitory effect can be reversed upon the interaction of the nSH2 domain of p85 with phosphorylated Tyrosine Kinases. This suggests that activation by Ras must be subsequent to the interaction of the PI3K complex interaction with Tyrosine Kinases. Blocking the p110α-Ras interaction through genetic mutation of the *Pi3kca* gene in mice has resulted in defective lymphatic vessel development, often leading to perinatal death, and significantly reduced rates of endogenous Ras oncogene-induced tumorigenesis when perinatal death is not observed, implicating Ras-binding to p110α as a requisite for Ras-driven tumorigenesis (Gupta et al. 2007). Recent work shows that blocking the p110α-Ras interaction in EGFR-mutant-driven lung tumors inhibits tumor onset and induces regression of established tumors in mouse models (Murillo et al. 2018). This finding suggests that targeting the Ras-PI3K interaction

may be a promising alternative to tyrosine kinase inhibitors, the effects of which can be diminished by the development of inhibitor resistance, in lung cancer therapy.

5.1.1 Dysregulation of PI3Kα in Human Cancers

A link between PI3Kα activity and cancer was initially suggested through several observations: its kinase activity can be associated with viral oncoproteins, mutations in the tumor-suppressor protein PTEN result in constitutive activation of PI3K (Li et al. 1997), and amplification of the genes encoding p110α (*PIK3CA*) and protein AKT are present in various cancers (Chang et al. 1997; Zhao and Vogt 2008a). Continued investigations showed amplification or "hot-spot" mutation of *PI3KCA* in solid tumors such as breast, cervical, endometrial, and ovarian cancers (Samuels et al. 2004; Bertucci et al. 2019; Isakoff et al. 2005; Parsons et al. 2005; Vasan et al. 2019). It has been reported that approximately 32% of colon cancers, 27% of glioblastomas, 25% of gastric cancers, between 8 and 40% of breast cancers, and 4% of lung cancers exhibit mutations in *PIK3CA*, emphasizing the functional importance of p110α in human cancers (Samuels et al. 2004). Such genetic alterations consist exclusively of somatic missense mutations and occur non-randomly, clustered to certain sites. In vitro and in vivo experiments show *PIK3CA* mutations result in increased lipid kinase activity (Kang et al. 2005; Bader et al. 2006). Of the three most common mutation sites in *PIK3CA* (E542, E545, and H1047), E542 and E545 are located in the helical domain while H1047 is located in the kinase domain. Approximately 47% of mutations in *PIK3CA* occur in the helical domain, 33% in the kinase domain, and 8% in the ABD (Samuels et al. 2004). These oncogenic mutations weaken the interactions that stabilize the inhibited p110α-p85α heterodimer (Echeverria et al. 2015). The high frequency of *PIK3CA* mutations in the helical domain is highlighted by the network of hydrogen bonds observed between the nSH2 and kinase loops residues, as well as with the helical domain residues that would be disrupted upon nSH2 movement when it binds phosphorylated receptors (Miller et al. 2014). Molecular dynamics studies have demonstrated that changes in protein dynamics at these interfaces can increase the population of catalytically competent states (Chakrabarti et al. 2020). Furthermore, the helical-nSH2 interface is the binding site of allosteric binding fragments, highlighting the dynamic role of this area and its role in pYXXM-mediated activation (Miller et al. 2017). Mutations in the regulatory subunit, p85, can weaken the inhibitory interaction to p110α (Sun et al. 2010). A large number of mutations distributed throughout *PIK3R1* (the gene encoding p85α) are present in endometrial cancers. Contrary to the hot-spot p110α mutations, p85α mutations tend not to be clustered (Cheung et al. 2011).

In accordance with the prevalence of *PIK3CA* mutations in human cancers, p110α remains at the forefront of clinical research. In 2019, a p110α-specific inhibitor, Alpelisib, was approved in the U.S. for the treatment of a subset of patients with advanced or metastatic breast cancer with mutations in the *PI3KCA* gene (Andre et al. 2019).

5.2 PI3Kβ

Similar to PI3Kα, PI3Kβ is expressed in most cell types (Hu et al. 1993; Utermark et al. 2014). The isoform-specific roles of PI3Kβ are complex and remain poorly understood. However, a number of observations in both cellular and organismal models have been made regarding activities associated with this isoform, which is the only class I PI3K that signals downstream of both RTKs and GPCRs (Kubo et al. 2005; Kurosu et al. 1997; Tang and Downes 1997). Although PI3Kβ has been suggested to have a functionally overlapping role with PI3Kγ in transducing signals downstream of GPCRs (Guillermet-Guibert et al. 2008), it is still essential for development, evidenced by the fact that, in mice, a homozygous allele deletion of this isoform can cause embryonic lethality within 3.5 days (Bi et al. 2002). However, *PIK3CB* gene knockout in other models permits a small population of embryos to survive despite significant lethality (Guillermet-Guibert et al. 2015). In conditional or heterozygous PTEN mouse knockout models of prostate cancer, the removal or inactivation of PI3Kβ was found to prevent PTEN loss-induced tumorigenesis, consistent with in vitro results that demonstrated inhibition of growth in PTEN-deficient human cancer cell lines upon PI3Kβ downregulation (Jia et al. 2008; Ni et al. 2012; Wee et al. 2008). Clinical trials using PI3Kβ-selective inhibitors to treat patients with PTEN-deficient solid tumors were also conducted to explore the potential viability of this observation (Bédard et al. 2018). However, other factors, including tissue type and genomic context, have been shown to shift this isoform-dependent effect to PI3Kα or even involve other isoforms (PI3Kδ and PI3Kγ) instead, suggesting that it may be a phenomenon of isoform-specific tissue expression (Schmit et al. 2014; Subramaniam et al. 2012). Potential evidence for PI3Kβ-mediated oncogenesis in HER2 models of breast cancer has also been reported (Ciraolo et al. 2008; Utermark et al. 2012), but the distinct role of this isoform in cancer remains unclear. Sequence and functional analysis of the wildtype PI3Kβ p110 subunit has suggested that it is analogous to the well-studied p110α N345K mutant of PI3Kα, which has enhanced oncogenic potential due to a disruption in the inhibitory C2-iSH2 interface (Dbouk et al. 2010). In the context of cell signaling, the p110β isoform has been pursued as a target for anti-thrombotic therapies (Jackson et al. 2005; Martin et al. 2010), due to its effects on both the Rap1 GTPase via Rasa3 and on AKT phosphorylation and GSK3 activity in platelets (Battram et al. 2017; Laurent et al. 2015; Moore et al. 2013; Stefanini et al. 2015), although the details of this signaling pathway remain unclear. There is also evidence that it may be implicated in mediating the production of reactive oxygen species (ROS) in neutrophils upon simultaneous activation by IgG-antigen complexes and leukotriene B_4, which interacts with the BLT1 receptor (Houslay et al. 2016; Kulkarni et al. 2011).

5.3 PI3K*δ*

By far, the best characterized PI3Kδ complex is the one comprising p110δ and p85α. While p110δ displays preferential selectivity for p85α over p85β, there are no available data to quantitate the relative occurrence of each PI3Kδ heterodimer combination or the relative binding affinities of p110δ for the two different regulatory subunits (Tsolakos et al. 2018). The tissue distribution of p110δ is restricted to the hematopoietic system and multipotential progenitors (Hemmati et al. 2019), as suggested by expressed sequence tag analysis. This is in contrast to the ubiquitously expressed p110α and p110β (Sawyer et al. 2003). In agreement with this observation of its enrichment in the hematopoietic system, PI3Kδ has been shown to regulate many aspects of the immune system including lymphocyte cell growth, proliferation, and differentiation (Fig. 5). Importantly, unlike the embryonic lethality of p110α and p110β deletions, p110δ deletion, like p110γ deletion, can produce viable phenotypes

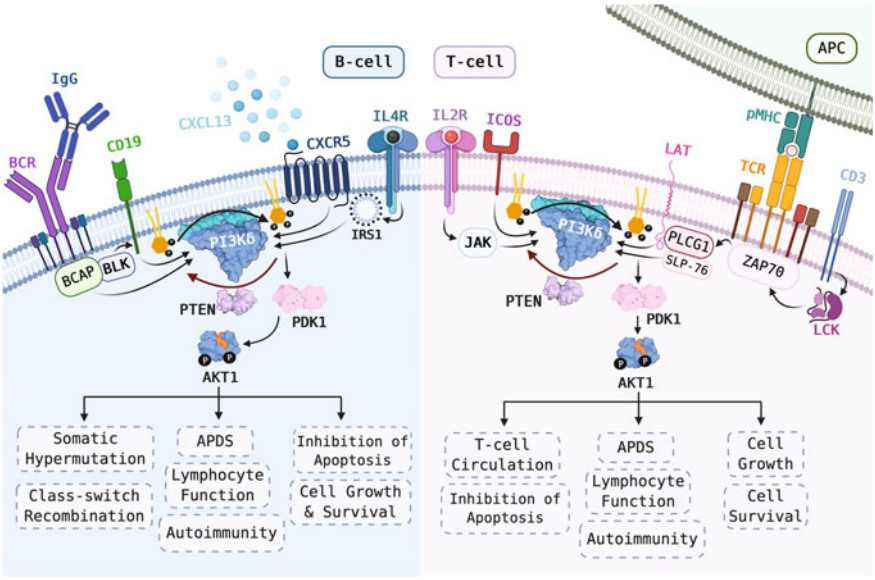


Fig. 5 B-cell and T-cell receptor PI3Kδ signaling. Left: In B-cells, PI3Kδ gets activated via the CXCR5 or IL-4R through sensing of chemokines such as CXCL13 or IL-4, respectively. The phosphorylated motifs of CD19 or BCAP are recognized by the SH2 domain of p85α. In a manner similar to PI3Kα, PI3Kδ can signal downstream of IL-4R using IRS1. Right: In T-cells, PI3Kδ gets activated by recognizing ICOS phosphorylation which triggers ICOS-mediated co-stimulation; whether ZAP70 and LAT are involved is not completely known. The activation through IL2R concomitant with JAK kinase is being actively studied. This rendition was created in BioRender using the built-in icons or generating icons from the deposited structures of PI3Kδ (PDB ID 4OVU), AKT1 (PDB ID 6NPZ), PTEN (PDB ID 1D5R), and PDK1 (PDB ID 1HW1). The structure of PI3Kα (PDB ID 4OVU) was used for PI3Kδ since they are of sufficient homology at this resolution

(Vanhaesebroeck et al. 2005). Activation of PI3Kδ is directly or indirectly regulated by receptors including B-cell and T-cell, cytokine, toll-like, and Fc receptors.

5.3.1 Regulation of Adaptive Immune Response by PI3Kδ

As with other class IA PI3Ks, PI3Kδ can be activated by the binding of the SH2 domains in the regulatory subunit with phosphorylated YXXM (pYXXM) motifs found in upstream cell receptors and receptor-associated proteins. As with other PI3K isoforms and the cells they act in, phosphorylation of AKT following PDK1 recruitment by PIP_3 leads to the upregulation of transcription factors that translocate to the nucleus and increase the rate of transcription of genes involved in apoptosis and cell cycle regulation (Shymanets et al. 2015). In B cells, PI3Kδ can be activated through several mechanisms: (1) B Cell Receptor (BCR) crosslinking, (2) stimulation of Interleukin-4 receptor (IL4R) by Interleukin-4 (IL4), and (3) by the G-protein coupled receptor (GPCR) CXCR5 (Fig. 5) (Lucas et al. 2016). Binding of B-Cell Associated Protein (BCAP) (a pYXXM motif-containing protein) to the SH2 domain of the regulatory p85α subunit is one mechanism underlying PI3Kδ activation by BCR crosslinking (Okada et al. 2000). In another PI3Kδ activation mechanism by BCR crosslinking, BCR antigen engagement promotes tyrosine phosphorylation of the immunereceptor tyrosine-based activation motif (ITAM). In this mechanism, BCR co-opts CD19 (a pYXXM motif-containing protein) (Burger and Chiorazzi 2013; Lucas et al. 2016; Nunes-Santos et al. 2019). Alternatively, IL4R stimulation results in the recruitment of IRS1, which also contains a pYXXM motif, enabling binding to the regulatory subunit (Lucas et al. 2016). The mechanism of PI3Kδ activation by CXCR5 is still unknown.

As observed in B cells, PI3Kδ can be activated in T cells by stimulation of Interleukin-2 receptor (IL2R) through Interleukin-2 (IL2) (Lucas et al. 2016). Rather than IRS1, the intermediate effector protein between IL2R and PI3Kδ is JAK, which also contains a YXXM motif (Lucas et al. 2016). Additionally, PI3Kδ can be activated in T cells by Inducible T-cell Co-stimulator (ICOS) engagement, which contains a YXXM motif, and by LAT following LAT phosphorylation mediated by ZAP70, a molecule that is part of the signaling downstream of the T cell receptor complex (Lucas et al. 2016). The specific mechanism by which LAT interacts with PI3Kδ remains unknown.

The extensive literature on pathways downstream of PI3Kδ suggests that this isoform plays a diverse set of roles in immune cell functions in health and disease. In hematopoietic stem cells, p110δ has been reported to play a dispensable role in repopulation, a redundant role to p110α in hematopoiesis, and an essential role in multilineage reconstitution (Hemmati et al. 2019). In neutrophils, PI3Kδ participates in trafficking and directional movement (Puri et al. 2004; Thomas et al. 2009). In T cells, p110δ deletion results in a disruption of T follicular helper cell development which in turn attenuates T cell dependent responses in B cells, including underregulated induction of the processes underlying affinity maturation and somatic hypermutation and the process through which B cells express different heavy-chain constant

regions, i.e., class switch recombination (Rolf et al. 2010). In T follicular helper cells, PI3Kδ has been suggested to be a major effector downstream of ICOS signaling that is critical for T follicular helper cell development (Preite et al. 2018; Rolf et al. 2010). In B cells, p110δ activity is critical for DNA synthesis, phosphorylation of several key enzymes including AKT, and a component of intracellular Ca^{2+} mobilization that is PI3K-dependent (Bilancio et al. 2006). Additionally, inactive p110δ in resting B cells has been shown to result in the failure of the resting B cells to enter the cell cycle (Bilancio et al. 2006).

PI3Kδ dysregulation has been linked to several chronic inflammatory diseases including asthma and arthritis, and immunodeficiency, such as Activated Phosphoinositide 3-Kinase δ Syndrome (APDS) (Fig. 5). In many cases, PI3Kδ deactivation through inhibition or deletion plays a beneficial role. For example, in asthma, a chronic inflammatory disease that has been linked to PI3Kδ dysregulation, several groups have reported that PI3Kδ genetic inactivation or small molecule inhibition results in attenuated immune hyper-responsiveness to airway constriction-causing agents such as methacholine and ovalbumin (Nashed et al. 2007; Park et al. 2010). Similarly, in chronic obstructive pulmonary disease, PI3Kδ inhibition shows anti-oxidative stress properties by reducing the activity of histone deacetylase 2, an enzyme that perpetuates inflammatory processes in cigarette smoking-induced oxidative stress (To et al. 2010). Mouse models of arthritis have shown that genetic deletion or inhibition of PI3Kδ can result in reduced joint erosion (Randis et al. 2008). APDS, also known as "p110δ-activating mutation causing senescent T cells, lymphadenopathy, and immunodeficiency" (PASLI disease), is a rare autosomal dominant immunodeficiency that can result from gain-of-function mutations in the *PIK3CD* gene encoding the p110δ catalytic subunit (APDS1) or from mutations in the *PIK3R1* gene encoding p85α and its splice variants (APDS2). Patients with either type of APDS have increased susceptibility to bacterial and viral infection and dysregulation of the PI3K/AKT pathway that can lead to lymphoproliferation and autoimmunity (Durandy and Kracker 2020; Singh et al. 2020). Increased predisposition to B cell lymphoma has also been observed as a result of APDS (Durandy and Kracker 2020). The most common mutation in APDS1 patients, an E1021K substitution (in the kinase domain), results in increased kinase activity and membrane associativity of p110δ (Lucas et al. 2014). In APDS2 patients whose *PIK3R1* mutation leads to the truncation of the p110 binding domain in the p85α subunit, the p85α regulatory subunit cannot bind to the p110δ catalytic subunit, resulting in underregulated elevated phosphorylation by the p110δ subunit (Deau et al. 2014).

5.3.2 PI3Kδ Signaling Downstream of Toll-Like Receptors and FcRs

In addition to being activated by Toll-Like (TLRs) and Fc Receptors (FcRs), along with the other PI3K isoforms, PI3Kδ serves as a key regulator of the signaling cascades coordinated by these receptors. Early studies suggested isoform non-specific, Rac 1-dependent activation of PI3Ks by TLRs and, simultaneously, PI3K-mediated inhibition of TLR signaling pathways (Arbibe et al. 2000; Fukao and

Koyasu 2003; Sarkar et al. 2004). Since phosphorylation of tyrosine residues on TLR2, TLR3, and TLR8 is a requirement for PI3K recruitment, it has been suggested that PI3K activation by TLRs occurs as a result of direct linkage between phosphorylated tyrosine residues in TLRs (Sarkar et al. 2004; Rajagopal et al. 2008). In better defining the specific roles of the PI3K isoforms, it has been shown that p110δ is a negative regulator of the signaling cascades initiated by TLR ligands (Dil and Marshall 2009). Namely, p110δ genetic inactivation results in significantly reduced B cell proliferation in response to lipopolysaccharide, which associates with TLR4, or deoxycytidyl-deoxyguanosine-containing microbial DNAs, which associate with TLR9. p110 inhibitors (e.g., Idelalisib) effect a similar reduction in B cell proliferation in a dose-dependent manner. Furthermore, inactivation and inhibition of p110δ also result in an altered pattern of TLR9-induced antibody isotype switch, differential regulation of TLR-mediated B cell cytokine production, impaired TLR4-mediated antiviral response, and a significant reduction in TLR-stimulated Interferon-α (IFN-α) production in plasmacytoid dendritic cells, the primary producers of IFN in response to TLR-stimulation (Dil and Marshall 2009; Guiducci et al. 2008). More recently, PI3Kδ has been suggested to play a significant role in the antiviral response to the West Nile Virus (WNV) by modulating the induction of type I interferon in human cell lines, embryonic fibroblasts, and mouse macrophages (Wang et al. 2017). In this case, p110δ inhibition decreased type I IFN production and increased WNV replication significantly more than inhibition of the remaining class I isoforms, suggesting that p110δ is the dominant isoform for type I IFN induction in this disease model. In agreement with the abovementioned function of PI3Kδ in the immune response against WNV, studies by Uzonna and colleagues have established that host PI3Kδ signaling plays a central role in the progression of *Leishmania* infection. When treated with p110δ inhibitors, mice infected with *Leishmania* showed reduced parasite load and cutaneous lesions suggesting p110δ inhibition suppresses infection (Khadem et al. 2017).

PI3Ks can be activated through FcRs (Vanhaesebroeck et al. 2001, Ben Mkaddem et al. 2019). While not completely understood, the underlying mechanism is thought to involve the recruitment of Src-kinase family members that follows FcR engagement with immune complexes and results in the tyrosine phosphorylation of the ITAM motif and subsequent activation and recruitment of Syk. In particular, PI3Kδ regulates the inflammatory effects of the Fcγ class of Fc receptors. Genetic deletion of p110δ has been shown to result in resistance to acute IgG immune complex-induced lung injury, a disease coordinated by FcγR activation (Konrad et al. 2008). More generally, recent evidence suggests that FcγR-PI3Kδ drives a pro-apoptotic signaling pathway in neutrophils in which PI3Kδ modulates downstream effectors upon the ligation of FcγRs (Chu et al. 2016). In human mast cells, genetic or pharmacological inactivation of p110δ alters type I FcεR-coordinated degranulation and cytokine release in vitro (Ali et al. 2004).

5.3.3 Therapeutic Application of PI3Kδ Inhibitors

Early studies identified several PI3K inhibitors as potentially effective therapeutic agents for targeting immune cell signaling, but the uniform inhibition of different PI3K isoforms and non-PI3K lipid kinases by these inhibitors resulted in off-target effects (Powis et al. 1994; Liu et al. 2005; Gharbi et al. 2007). Given that expression of PI3Kδ is largely restricted to the hematopoietic system, p110δ inhibitors present an opportunity to circumvent this issue and selectively target signaling in leukocytes. To date, idelalisib, a highly selective p110δ inhibitor, and copanlisib, a PI3K inhibitor with ten-fold increased affinity for p110α and p110δ over the other two class I isoforms, have been approved in the U.S. to treat B cell malignancies and relapsed follicular lymphoma, respectively (Markham 2014; Krause et al. 2018). Interestingly, investigations in response to idelalisib-resistant tumors have shown that IGFR-1 inhibition can be used to overcome resistance to p110δ inhibitors, providing some evidence for signaling activity between PI3Kδ and IGFR-1 (Scheffold et al. 2019). Several other p110δ inhibitors are currently under research, following the lead of idelalisib and IC87114, an inhibitor with 58-fold more selectivity for p110δ than p110γ, and 100-fold more selectivity for p110δ than either p110α or p110β. These inhibitors have been shown to reduce pro-inflammatory cytokine production in memory T cells in mice, suggesting potential therapeutic value in treating autoimmune and inflammatory diseases (Soond et al. 2010).

Dual inhibition of p110δ and p110γ has been found to be a more effective therapeutic option than single p110δ inhibition for several disease models. For example, in vivo arthritis models show that while p110δ inhibition results in some reduction in joint destruction and erosion, dual p110δ and p110γ inhibition results in significant reductions along with reduced neutrophil transmigration (Randis et al. 2008; Rodrigues et al. 2019). Accordingly, although PI3Kδ inhibition shows a weakly suppressive effect on inflammatory marker proteins such as TNF-α, IL-1α, and PGE2, dual p110δ/γ inhibition shows enhanced inflammatory marker suppression in human cells (Williams et al. 2010). In this regard, duvelisib, a dual p110δ/γ inhibitor, has been recently approved in the U.S. for the treatment of relapsed or refractory chronic or small lymphocytic leukemia (Randis et al. 2008; Rodrigues et al. 2019).

5.4 PI3Kγ

Even though it was originally reported to be expressed solely in immune cells, PI3Kγ is expressed in most human tissues including endothelial cells, microglia, neurons, heart, and specialized cells in the kidney, pancreas, and prostate (Stephens et al. 1994b; Stoyanov et al. 1995b; Becattini et al. 2011; Costa et al. 2011; Konig et al. 2010; Narita et al. 2002; Patrucco et al. 2004; Voigt et al. 2006). PI3Kγ is found as two different functional heterodimers; the catalytic subunit, p110γ, either associates with the regulatory subunit p101 or with p84. In contrast with the regulatory subunits of class IA, p101 and p84 potentiate activation of PI3Kγ. Also, in contrast to p85,

the adaptor protein of class IA, p101 is unstable when expressed in mammalian or insect cells (Lopiccolo et al. 2015; Brock et al. 2003; Voigt et al. 2006; Tsolakos et al. 2018). The lack of structural information about p101 is probably a result of this characteristic (Brock et al. 2003). The product of the gene *PIK3R6*, p84, was discovered by homology with p101 and is distinctly stable in heterologous expression systems. Although p101 is found mainly in the nucleus, this localization shifts to the cytoplasm upon coexpression with p110γ (Tang and Downes 1997). The regulatory subunit p84 is strongly expressed in the heart, the central nervous system, and the immune system. The complex of p110γ with p84 dissociates, exchanges partner subunits (Shymanets et al. 2013), and displays reversible dimerization. Moreover, the phosphorylation of the helical domain of p110γ promotes p101γ-p84 complex disassociation, as has been observed in mast cells and in in vitro assays (Walser et al. 2013).

The two known PI3Kγ heterodimers display different and independent signaling cascades (Schmid et al. 2011; Brazzatti et al. 2012) according to their respective tissue distribution, which distinguishes their functions (Bohnacker et al. 2009; Shymanets et al. 2013). Antibodies against each complex have helped dissect their specific roles (Shymanets et al. 2015): p110γ-p84 is constitutively expressed, while the p110γ-p101 is upregulated upon activation (Shymanets et al. 2015). Monoclonal antibodies (mABs) that block the heterodimer formation reduce the basal lipid kinase activity of both PI3Kγ heterodimers in a concentration-dependent manner with a preference for p110γ-p84. PI3Kγ achieves maximal activation by the interaction of its regulatory subunit p101 with Ras (Stephens et al. 1997; Suire et al. 2005). Although free p110γ is not localized to the plasma membrane in vitro, it is sensitive to GPCR activation. Interestingly, p110γ-p101 binding affinity to any of the Gβγ dimers is five-fold higher than that of p110γ alone (Stephens et al. 1997). In contrast with p110α, p110γ is stable in the absence of its regulatory subunits (Nurnberg and Beer-Hammer 2019; Rynkiewicz et al. 2020).

Analogously to p110α, p110γ displays activity as a protein kinase and autophosphorylates at Ser 1101 (Maheshwari et al. 2017; Czupalla et al. 2003). On the other hand, unlike other class IA PI3Ks, p110γ autophosphorylation does not affect its lipid kinase activity (Czupalla et al. 2003). Phosphorylation of other substrates by p110γ is less studied, as is the biological relevance of PI3Kγ protein kinase activity. In non-muscle tropomyosin, one of the few PI3Kγ-identified substrates, phosphorylation regulates β-adrenergic receptor density in the heart (Li et al. 2017, Naga Prasad et al. 2005).

5.4.1 Activation by GPCRs and Ras

PI3Kγ, together with class IA PI3Kβ, is activated by the release of Gβγ complexes of G-protein Coupled Receptors (GPCR). G-proteins are prenylated with either C20 or C15 and attached to the inner layer of the lipid membrane, which brings PI3K to the membrane and closer to the substrate (Suire et al. 2012). Variation in PI3Kγ

activation due to different G-protein prenylation lengths continues to be a topic of investigation (Vadas et al. 2013; Dbouk et al. 2012; Maier et al. 1999, 2000).

Interestingly, prenylated Ras variants further activate PI3Kγ by binding of H-, N- or K-Ras to the RBD of p110γ. The X-ray crystallographic structure of the complex of p110γ with H-Ras G12V (PDB ID 1HE8) used mutations at residues 80 and 186 to make the interaction more stable, and in this way identified the binding site. The K_D of p110γ for binding to RAS-GTP is around 3 μM (Pacold et al. 2000), whereas the affinity of the oncogenic mutant G12V-H-Ras is about 20-fold tighter (lower K_D). H-Ras stimulates both p110γ-p101 and p110γ-p84 in vitro, but mutation of the RBD only suppressed p110γ-p84 activation (Kurig et al. 2009). Besides the difference in activation, the p110γ-p84 heterodimer plays roles that are independent of its lipid kinase activity. For example, some scaffolding roles have been described for the p110γ-p84 complex with PDE3b contributing to regulation of cell adhesion in arterial cells of the cardiovascular system as well as inflammatory response syndrome (Perino et al. 2011; Rynkiewicz et al. 2020). Other regulators of PI3Kγ are BAR kinase, PKA, and P2A (Vadas et al. 2013).

It has been shown that in neutrophils, RAS does not activate either of the PI3Kγ complexes in the absence of GPCR signaling (Rynkiewicz et al. 2020). Interestingly, in vitro activation assays of p110γ by GTP-RAS have not recapitulated the in vivo observation.

5.4.2 PI3Kγ as a Therapeutic Target

PI3Kγ has been targeted as anti-inflammatory and autoimmune therapies since the beginning of this century, but recently its role in cancer and metabolic disease has opened a new frontier. Reducing levels of PI3Kγ inhibits oncogenesis. Moreover, PI3Kγ inhibitors provide immuno-stimulatory activity to macrophages and thereby promote antitumor immunity (De Henau et al. 2016; Kaneda et al. 2016). In contrast to the beneficial activation of PI3Kα in cardiac growth, PI3Kγ and δ activation contribute to inflammation of the heart upon reperfusion. Activation of PI3Kγ is detrimental to cardiomyocyte contractility via the PDE. p110γ knock out mice had enhanced contractility and mice expressing a kinase-dead p110γ showed reduced hypertrophy and fibrosis (Becattini et al. 2011; Costa et al. 2011; Konig et al. 2010; Narita et al. 2002; Patrucco et al. 2004; Voigt et al. 2006; Damilano et al. 2011). Furthermore, the upregulation of PI3Kγ in cardiomyocytes under stress, which in turn triggers cardiac remodeling has pushed its evaluation as a candidate protein for inhibition in cardiovascular disease (Damilano et al. 2011; Perino et al. 2011). Specifically, the p110γ/p84 complex has been shown to be involved in cardiac inflammation (Li et al. 2017). Following these discoveries, PI3Kγ has been a discovery target to block pro-inflammatory processes in order to reduce ischemia/reperfusion injury, inhibit rheumatoid arthritis, and treat lupus nephritis (Doukas et al. 2006; Rommel et al. 2007). Several inhibitors have been approved for clinical evaluation; IPI-549, Duvelisib, and Tenalisib have been reviewed in Miller et al. (2019).

6 From Molecular Determinants to Frequency of Signal and Reaction Rates

The nodes of the PI3K pathway have been identified in the last 30 years but it still remains unclear how different environmental clues determine the activation outcome. For example, what input is required for the multitude of responses from glucose uptake and proliferation to cell death in others? PI3Kα inhibitors have failed to deliver the expected cures due to toxicity or resistance (Fruman et al. 1998). On the other hand, selective inhibitors have proven useful for treatment of *PIK3CA* overgrowth spectrum (Venot et al. 2018, 2019) and PI3K dysregulation such as Activated PI3Kδ syndrome (APDS) (Rao et al. 2017).

Hormones and growth factors, such as insulin and platelet-derived growth factor, bind to their specific receptor tyrosine kinases which dimerize and cross-phosphorylate in their cytoplasmic regions. The phosphorylation sensed by the p110-associated adaptor protein is the initial activation of the pathway that can get further boosted by RAS (PI3Kδ and PI3Kγ), RAC/CDC42 (PI3Kβ), and G-Protein-Coupled Receptors (PI3Kβ). These signals, together with allosteric modulators, reaction rates, types of cells, duration of the signal, and frequency, may lead to different outputs and phenotypes (Madsen and Vanhaesebroeck 2020; Albeck et al. 2013).

In conclusion, the four PI3K isoforms in class I, which catalyze the phosphorylation of PIP_2, exhibit non-overlapping, diverse roles in the cell. The isoforms, which are delineated into class IA and IB on the basis of the regulatory subunit, share significant structural similarities in the p110 catalytic subunit. Upon receptor activation, PI3Ks engage in pathways that regulate cellular activities, including differentiation, growth, proliferation, motility, and survival. Dysregulation of each PI3K isoform underlies diseases such as cancer (PI3Kα, PI3Kγ, PI3Kβ), chronic inflammation and immunodeficiency (PI3Kδ, PI3Kγ), and metabolic disease (PI3Kγ). Efforts to better understand PI3K pathways, the intensity of their signals, and molecular mechanisms of action alongside developing therapeutic agents continue to be at the forefront of the field.

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An Overview of Class II Phosphoinositide 3-Kinases

Emily Yan Zhi Heng and Tania Maffucci

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Abstract Phosphoinositide 3-kinases (PI3Ks) catalyse the synthesis of specific members of the family of lipids collectively known as 'phosphoinositides'. These PI3Ks products can in turn modulate activation of many downstream proteins, ultimately regulating several cellular processes. Mammalian cells possess eight PI3Ks which are grouped into three classes based on their structure and substrate specificity. While class I and III PI3Ks have been extensively investigated, our understanding of the three class II members has only improved in most recent years. This chapter will summarise some of the available information on mammalian class II PI3Ks and their physiological roles.

E. Y. Z. Heng · T. Maffucci (✉)
Centre for Cell Biology and Cutaneous Research, Blizard Institute, Faculty of Medicine and Dentistry, Queen Mary University of London, 4 Newark Street, London E1 2AT, UK
e-mail: t.maffucci@qmul.ac.uk

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List of Abbreviations

DAG	Diacylglycerol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
HCC	Hepatocellular carcinoma
IL	Interleukin
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MEF	Mouse embryonic fibroblast
NADPH	Nicotinamide adenine dinucleotide phosphate
OA	Osteoarthritis
PI3K	Phosphoinositide 3-kinase
PtdIns	Phosphatidylinositol
PtdIns3*P*	Phosphatidylinositol 3-phosphate
PtdIns4*P*	Phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4)P_2	Phosphatidylinositol 3,4-bisphosphate
PtdIns(3,4,5)P_3	Phosphatidylinositol 3,4,5-trisphosphate
PX	Phox homology
RA	Rheumatoid arthritis
TNF	Tumour necrosis factor

1 Introduction

Phosphoinositide 3-kinases (PI3Ks) are a conserved family of lipid kinases that catalyse the phosphorylation of specific phosphoinositides (Cantley 2002; Vanhaesebroeck et al. 2012; Maffucci 2012), a group of lipids consisting of a *myo*-inositol headgroup linked to a diacylglycerol (DAG) through a phosphodiester bond (Fig. 1). Specifically, PI3Ks catalyse phosphorylation on position three within the *myo*-inositol ring of phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], leading to the synthesis of phosphatidylinositol 3-phosphate (PtdIns3*P*), phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], respectively (Vanhaesebroeck et al. 2001; Domin and Waterfield 1997) (Fig. 1). These lipids in turn regulate the activation of several proteins, ultimately controlling a plethora of intracellular functions (Vanhaesebroeck et al. 2010, 2016; Engelman et al. 2006; Ghigo et al. 2012; Falasca and Maffucci 2007, 2012; Gulluni et al. 2019; Bilanges et al. 2019). Eight mammalian PI3Ks exist and they were grouped into three classes based on their structure and substrate specificity (Vanhaesebroeck et al. 2001; Domin and Waterfield 1997). Class I PI3Ks, by far

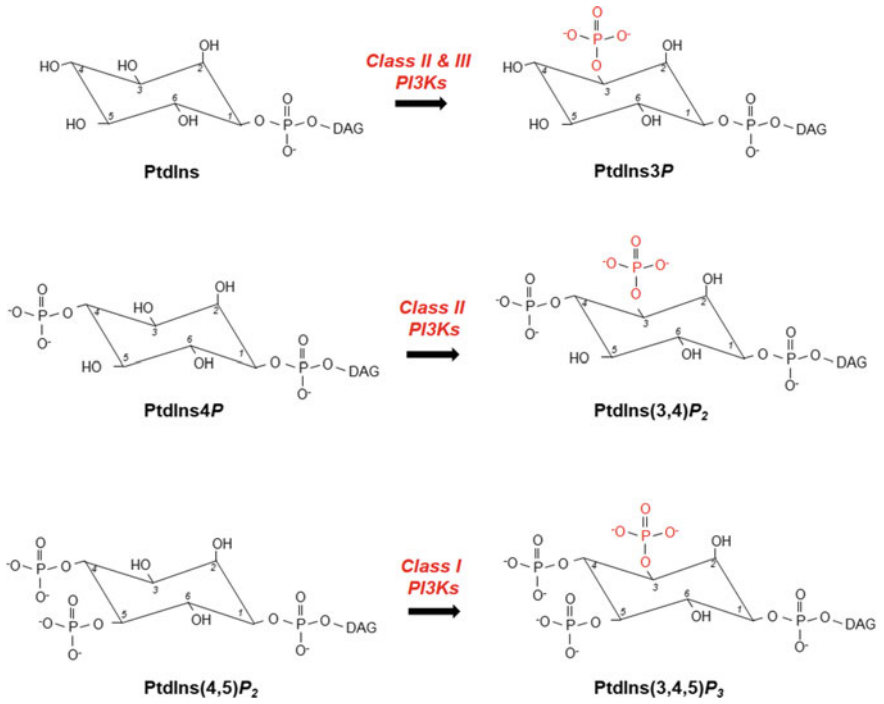

Fig. 1 Schematic representation of the lipid substrates and products of the distinct classes of PI3Ks. Positions within the inositol ring are indicated by numbers

the best characterised amongst the members of this family, are dimers of a regulatory and one of four catalytic subunits, and are mainly responsible for synthesis of PtdIns(3,4,5)P_3 in vivo. Class II PI3Ks have been reported to catalyse synthesis of both PtdIns3*P* and PtdIns(3,4)P_2. Class III PI3K catalyses synthesis of the bulk of intracellular PtdIns3*P* (Fig. 1).

For a long time, class II PI3Ks have been the least investigated amongst all PI3Ks but our understanding of these enzymes has massively improved in most recent years. This chapter will focus on mammalian class II PI3Ks and their physiological roles. It must be noted, however, that accumulating data in literature also points to a role for them in several human diseases (Vanhaesebroeck et al. 2016; Ghigo et al. 2012; Falasca and Maffucci 2012; Gulluni et al. 2019; Bilanges et al. 2019).

2 Identification and Tissue Distribution

A class II PI3K was first identified in *Drosophila melanogaster* (MacDougall et al. 1995). It was later discovered that class II enzymes are conserved from *Caenorhabditis elegans* to humans but they are not expressed in yeast [reviewed in Gulluni

et al. (2019), Bilanges et al. (2019), Margaria et al. (2019)]. Three members of class II PI3Ks exist in mammalian cells, namely PI3K-C2α, PI3K-C2β and PI3K-C2γ, encoded by the genes *PIK3C2A*, *PIK3C2B* and *PIK3C2G*, respectively. Human PI3K-C2α was cloned from U937 cells (Domin et al. 1997) while human PI3K-C2β was isolated from a cDNA library of MCF7 (Brown et al. 1997) and subsequently from U937 cells (Arcaro et al. 1998). Original studies indicated that PI3K-C2α was ubiquitously expressed with highest levels in heart, placenta and ovary (Domin et al. 1997). Expression of PI3K-C2β was shown to be high in thymus and placenta, while expression of PI3K-C2γ was identified in liver and prostate tissue as well as in breast and salivary glands (Rozycka et al. 1998; Ono et al. 1998). It is now well established that PI3K-C2α and PI3K-C2β are ubiquitously expressed while PI3K-C2γ shows a more restricted expression pattern (Consortium GTEx 2013). Interestingly, a recent study reported an increase of PI3K-C2β both at the mRNA and protein levels in fibroblasts obtained from individuals carrying homozygous loss-of-function mutations in *PIK3C2A* as well as upregulation of PI3K-C2β upon downregulation of PI3K-C2α in HeLa cells (Tiosano et al. 2019). Whether this is a compensatory effect or whether this study has unveiled a general mechanism of regulation of the expression levels of these two enzymes remains to be established. PI3K-C2γ levels can also be modulated, as suggested by a study reporting increased expression of this enzyme during liver regeneration following partial hepatectomy (Ono et al. 1998).

3 Structure of Class II PI3Ks

Class II PI3Ks are monomers of high molecular weight, possessing the PI3K core common to all family members (consisting of a C2 domain, a helical domain and the catalytic domain), a Ras binding domain (also found on the catalytic subunits of class I PI3Ks) but characterised by unique N-terminal and C-terminal extensions compared to the other PI3Ks (Fig. 2) (Vanhaesebroeck et al. 2010; Falasca and Maffucci 2012; Gulluni et al. 2019; Margaria et al. 2019). The C-terminal extension is common between the three class II PI3Ks and consists of a Phox homology (PX) domain and a C2 domain (Fig. 2). The N-terminal extensions differ between the three enzymes and allow some of them to bind to distinct proteins. For instance,

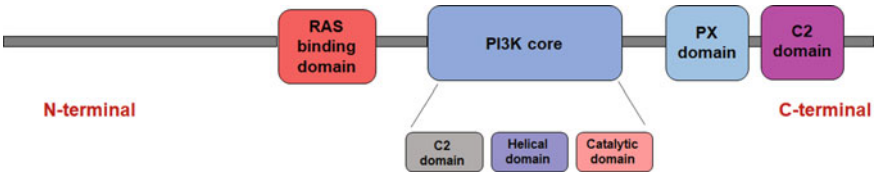

Fig. 2 Schematic representation of the common structure of class II PI3Ks, comprising an N-terminal region, a Ras binding domain, the PI3K core (consisting of a C2, a helical and a catalytic domain) and a common C-terminal, comprising a PX and a second C2 domain

both PI3K-C2α (Gaidarov et al. 2001) and PI3K-C2β (Wheeler and Domin 2006) can bind to clathrin via their N-terminal region, but PI3K-C2β can also interact with Raptor (Marat et al. 2017). In addition, PI3K-C2β possesses proline-rich motifs which have been investigated for their potential role in activation of the enzyme (Wheeler and Domin 2006), while PI3K-C2α can bind to transforming acidic coiled-coil-containing protein 3 (TACC3) via a region localised between the Ras binding domain and the PI3K core (Gulluni et al. 2017). Less is known about the structure of PI3K-C2γ which, in general, is the least characterised of the three enzymes, but it does not appear to possess a clathrin binding domain (Margaria et al. 2019).

4 Lipid Products

The differences between class II and class I PI3K enzymes were immediately clear, as indicated by an original study reporting that only 1% of the in vitro activity of PI3K-C2β was directed towards PtdIns(4,5)P_2 (Arcaro et al. 1998), the main substrate of class I PI3Ks. On the other hand, class II PI3Ks were found to catalyse phosphorylation of PtdIns and PtdIns4*P* (MacDougall et al. 1995; Arcaro et al. 1998, 2000; Gaidarov et al. 2001; Virbasius et al. 1996; Misawa et al. 1998), with PtdIns being the main substrate in vitro (Falasca and Maffucci 2012). While it was soon clear that PtdIns3*P* and, to a lesser extent, PtdIns(3,4)P_2, were the main products of class II PI3Ks in vitro, identification of their in vivo products was complicated by the absence of selective inhibitors for these enzymes. Indeed, data only started appearing following the advent of antisense/siRNAs/shRNAs-based techniques and, later on, with the generation of transgenic mice (Falasca et al. 2007; Maffucci et al. 2005; Wen et al. 2008; Boukhalfa et al. 2020a, b; Valet et al. 2015; Alliouachene et al. 2015; Franco et al. 2014; Yoshioka et al. 2012).

4.1 PtdIns3P

Over fifteen years ago, we reported that PI3K-C2α and PI3K-C2β catalyse the synthesis of PtdIns3*P* in vivo in response to insulin (Falasca et al. 2007) and lysophosphatidic acid (LPA) (Maffucci et al. 2005). These studies were amongst the first lines of evidence supporting a role for class II PI3Ks in the regulation of pools of PtdIns3*P* specifically synthesised in response to cellular stimulation. Consistent with this, PI3K-C2α-dependent pools of PtdIns3*P* were also detected in PC12 cells upon stimulation of exocytosis (Wen et al. 2008) and have been observed, more recently, in kidney epithelial cells in response to shear stress (Boukhalfa et al. 2020a, b). The possibility of a cell cycle-dependent, class II PI3Ks-mediated synthesis of this phosphoinositide was also supported by in vitro assays demonstrating that PtdIns3*P*, but not PtdIns4*P*, PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3, increased in the nuclei and nuclear

envelopes of HL-60 cells during transition into the G2/M-phase, likely due to activation of PI3K-C2β (Visnjić et al. 2003). Transgenic mouse models revealed that class II PI3Ks can also regulate pools of PtdIns3*P* in basal, unstimulated conditions, as detected in platelets from mice heterozygous for a catalytically inactive (kinase-dead) PI3K-C2α (Valet et al. 2015) or in unstimulated hepatocytes from a mouse model expressing a kinase-dead PI3K-C2β (Alliouachene et al. 2015). This latter study further indicated that PI3K-C2β modulation of PtdIns3*P* was cell type-specific, as levels of this phosphoinositide were reduced in hepatocytes but not in mouse embryo fibroblasts (MEFs) or splenocytes (Alliouachene et al. 2015).

Generally, class II PI3Ks have been implicated in localised synthesis of PtdIns3*P* within specific cellular compartments. For instance, the previously mentioned insulin- and LPA-dependent PtdIns3*P* was detected at the plasma membrane (Falasca et al. 2007; Maffucci et al. 2005). Similarly, PtdIns3*P* was specifically generated in large dense-core vesicles (Wen et al. 2008) or in the primary cilium area (Boukhalfa et al. 2020b) during exocytosis and in response to shear stress, respectively. Furthermore, MEFs lacking PI3K-C2α displayed reduced levels of a basal pool of PtdIns3*P* specifically localised around the base of the cilium but showed no alteration on this phosphoinositide within the rest of the cell (Franco et al. 2014). Similarly, selective ablation of PI3K-C2α in endothelial cells was shown to decrease the levels of PtdIns3*P* in endosomes (Yoshioka et al. 2012). Taken together these studies indicate that PI3K-C2α and PI3K-C2β can control synthesis of very localised pools of PtdIns3*P*, sometimes in response to specific cellular stimulation. To the best of our knowledge, there is no indication so far that PI3K-C2γ can also catalyse synthesis of PtdIns3*P* in vivo.

4.2 PtdIns(3,4)P_2

PtdIns(3,4)P_2 is the only in vivo product of PI3K-C2γ identified so far, and it appears to be localised specifically on Rab5-positive early endosomes (Braccini et al. 2015). Evidence that the other class II PI3Ks can also catalyse the synthesis of PtdIns(3,4)P_2 in vivo also exists, including the observation that transient downregulation of PI3K-C2α inhibited the insulin-induced synthesis of PtdIns(3,4)P_2, but not PtdIns(3,4,5)P_3 or levels of PtdIns3*P* in MIN6 pancreatic β cells (Leibiger et al. 2010) and more recent data indicating that PI3K-C2α and PI3K-C2β catalyse the synthesis of PtdIns(3,4)P_2 at late-stage endocytic compartments (Posor et al. 2013) and in lysosomes and late endosomes (Marat et al. 2017). Consistent with this, fibroblasts derived from patients displaying homozygous loss-of-function mutations in *PIK3C2A* revealed reduced PtdIns(3,4)P_2 overall and reduced PtdIns3*P* specifically at the ciliary base (Tiosano et al. 2019).

5 Mechanisms of Activation

Although our understanding of the physiological roles of class II PI3Ks has improved massively in the past decade, still little is known about the mechanisms of their activation (Falasca and Maffucci 2012; Bilanges et al. 2019). Association of class II PI3Ks to some growth factor receptors was reported (Falasca and Maffucci 2012), such as interaction of PI3K-C2α and PI3K-C2β with the epidermal growth factor receptor (EGFR) (Arcaro et al. 2000), of PI3K-C2α with the insulin receptor B (Leibiger et al. 2010), and of PI3K-C2β with the platelet-derived growth factor receptor (Arcaro et al. 2000) and c-Kit (Arcaro et al. 2002). This, however, has not led to a clear indication of whether such associations are part of a generic mechanism of activation of these enzymes, as well established for class I PI3Ks. In this respect, it is worth mentioning that binding of PI3K-C2α and PI3K-C2β to EGFR and ErbB-2 was already detectable in quiescent A431 cells although it increased upon EGF stimulation (Arcaro et al. 2000) and association of PI3K-C2β with c-Kit in small cell lung carcinoma cell lines did not appear to increase upon stem cell factor stimulation (Arcaro et al. 2002).

Original studies reported that the in vitro activity of PI3K-C2α increased upon removal of the region encompassing the clathrin-binding sites (Gaidarov et al. 2001) and that deletion of the proline-rich motifs of PI3KC2β affected its activity (Wheeler and Domin 2006), suggesting that the N-terminal extensions could be involved in regulation of their enzymatic activity. On the other hand, the involvement of the C-terminal PX and C2 domains in regulation of PI3K-C2α activation has been demonstrated by a recent study showing that these two domains fold onto the catalytic domain preventing its activity when the enzyme is in solution (Wang et al. 2018). Once the enzyme is recruited to the membrane, interactions of the N-terminal region with clathrin and of both PX and C2 domains with PtdIns(4,5)P_2 remove such an inhibition over the catalytic domain (Wang et al. 2018). Interestingly, Ca^{2+} has been reported to affect the interaction of PI3K-C2α PX-C2 domains with the membrane (Chen et al. 2018) and previous studies demonstrated that the activity of this enzyme could be increased by increasing concentration of Ca^{2+} (Wen et al. 2008) and by stimuli able to increase intracellular Ca^{2+} concentration (Wang et al. 2006). Currently, it is not known whether such an autoregulatory mechanism exists in all class II enzymes although it is worth mentioning that increased enzymatic activity of PI3K-C2β in vitro was observed upon deletion of the C-terminal C2 domain (Arcaro et al. 1998). Being the PX/C2 domains a characteristic feature of class II PI3Ks, it is tempting to speculate that this or similar mechanisms might regulate the activation of all class II PI3Ks.

Association with other proteins has been also proposed as a potential mechanism of activation (Falasca and Maffucci 2012), as in the case of PI3K-C2β and intersectin (Das et al. 2007). More recently, a study demonstrated that protein kinase N induces PI3K-C2β inactivation upon mitogen stimulation by promoting its association to 14-3-3 proteins (Wallroth et al. 2019). Evidence of post-translational modifications of PI3K-C2β is also present in the literature, from growth factor-induced tyrosine phosphorylation (Arcaro et al. 2002) to possible proteolysis (Visnjić et al. 2003) and

nitrotyrosylation (Chiang and Postlethwaite 2006). The specific role of these post-translational modifications in the modulation of class II PI3Ks enzymatic activity, however, remains to be clarified.

As discussed previously, an interesting aspect of class II PI3Ks biology is their ability to generate localised pools of PtdIns3*P* and PtdIns(3,4)P_2. Consistent with this, studies have reported intracellular relocation of class II enzymes as the way by which such localised synthesis of their products can be achieved. Examples include the insulin-induced translocation of a GFP-tagged PI3K-C2α to the plasma membrane of L6 cells (Falasca et al. 2007), the LPA-dependent translocation of a Myc-tagged PI3KC2β to the plasma membrane of HeLa and SKOV-3 cells (Maffucci et al. 2005) and the shear stress-induced relocation of PI3K-C2α to the primary cilium (Boukhalfa et al. 2020b).

Much work is still required to come to a full understanding of how the enzymatic activity of class II PI3Ks can be modulated in different cellular contexts.

6 Physiological Roles of Class II PI3Ks—Insights from Animal Models

6.1 PI3K-C2α

Our understanding of the physiological roles of class II PI3Ks has improved in the past ten years due to the generation of several mouse models, as recently summarised (Gulluni et al. 2019). Briefly, a critical role for PI3K-C2α during development was demonstrated by two studies reporting embryonic lethality upon full knockout of the enzyme, due to defects at the level of the primary cilium structure and function (Franco et al. 2014) and in vascular development and angiogenesis due to its key role in endothelial cells specifically (Yoshioka et al. 2012). Embryonic lethality was also reported in homozygous mouse models for either a kinase-dead (Alliouachene et al. 2016) or truncated versions (Mountford et al. 2015) of PI3K-C2α. Heterozygous mice from the kinase-dead model were viable and fertile although male mice presented early onset leptin resistance, mild age-dependent obesity, insulin resistance and glucose intolerance (Alliouachene et al. 2016). Heterozygous mice expressing the truncated versions of PI3K-C2α showed alteration of the internal membrane structure of platelets and impaired thrombosis (Mountford et al. 2015). These defects were not detected in platelets from PI3K-C2β deficient mice and were not enhanced in platelets from mice deficient in both enzymes (Petitjean et al. 2016), confirming a non-redundant role for PI3K-C2α in platelets.

6.2 PI3K-C2β

In contrast to PI3K-C2α, a PI3K-C2β full knockout mouse model was reported to be viable and fertile (Harada et al. 2005). This study focused in particular on the impact on the epidermis and reported no alteration in epidermal growth, differentiation or in the barrier function in knockout mice (Harada et al. 2005). Knockout mice were also shown to be resistant to passive cutaneous and passive systemic anaphylaxis (Srivastava et al. 2017). A mouse model expressing a kinase-dead PI3K-C2β revealed increased insulin sensitivity selectively in metabolic tissues as well as increased glucose tolerance and protection against liver steatosis induced by high-fat diet (Alliouachene et al. 2015).

6.3 PI3K-C2γ

A PI3K-C2γ global knockout mouse model was also reported to be viable and to develop normally. These mice showed reduced accumulation of glycogen in the liver and altered lipid metabolism and developed insulin resistance with age or upon high-fat diet (Braccini et al. 2015).

7 Physiological Roles of Class II PI3Ks—First Insight from Human Studies

A recent study reported the identification of the first monogenic disorder linked to mutations of a class II PI3K (Tiosano et al. 2019). The authors described five individuals from three unrelated consanguineous families showing similar clinical features, including short stature, cataracts, dysmorphic facial features, skeletal and teeth abnormalities. Most individuals also presented neurological abnormalities and secondary glaucoma. Hearing loss was also observed in some patients. Next-generation sequencing analyses showed that all affected family members, but none of the unaffected members, were homozygous for loss-of-function variants in *PIK3C2A* and indeed PI3K-C2α was not detected in patient-derived fibroblasts (Tiosano et al. 2019). This study indicated a critical role for PI3K-C2α in human development but it also highlighted a stark difference with the transgenic mouse models where lack of PI3K-C2α results in embryonic lethality. Whether this difference is due to different physiological roles in humans or potential compensatory effects of other PI3Ks (possibly PI3K-C2β, whose expression levels increase in fibroblasts from affected individuals (Tiosano et al. 2019)) remains to be established.

8 Cellular Functions Regulated by Class II PI3Ks

8.1 PI3K-C2α

Investigation into the molecular mechanisms responsible for the phenotypes detected in the different animal models revealed that PI3K-C2α is involved in several intracellular trafficking processes, including transport of cargos and signalling proteins to the primary cilium (Franco et al. 2014, 2016) as well as trafficking of vascular endothelial growth factor receptor-2 and sphingosine-1-phosphate receptor 1 (Yoshioka et al. 2012), vascular endothelial-cadherin (Yoshioka et al. 2012) and leptin receptor (Alliouachene et al. 2016). Studies using cell lines also indicated a role in clathrin-dependent (Gaidarov et al. 2001; Posor et al. 2013) and in dynamin-independent endocytosis (Krag et al. 2010) as well as in neurosecretory (Wen et al. 2008; Meunier et al. 2005) and insulin (Leibiger et al. 2010; Dominguez et al. 2011) granule exocytosis.

Data also indicate the involvement of PI3K-C2α in several different cellular processes, from regulation of vascular smooth muscle cell contraction (Wang et al. 2006; Yoshioka et al. 2007) to human cytomegalovirus replication (Polachek et al. 2016) and Kaposi's sarcoma-associated herpesvirus reactivation from latency (Abere et al. 2018). PI3K-C2α has been also implicated in regulation of autophagy (Merrill et al. 2017) through its ability to generate a localised pool of PtdIns3*P* which is critical during initiation of this cellular process (Nascimbeni et al. 2017a; Roberts and Ktistakis 2013). Recent data, in particular, have revealed a specific role for the PI3K-C2α-dependent PtdIns3*P* pool in shear stress-induced autophagy in kidney epithelial cells, as opposed to the PtdIns3*P* pool generated by class III PI3K which is required for activation of autophagy upon cellular starvation (Boukhalfa et al. 2020b).

A role for PI3K-C2α in insulin signalling was also suggested by data demonstrating that its downregulation reduced translocation of the glucose transporter GLUT4 to the plasma membrane and glucose uptake in muscle cells (Falasca et al. 2007) and it induced a switch toward mitogenic rather than metabolic signalling in pancreatic β cells (Leibiger et al. 2015). On the other hand, heterozygous mice for a kinase-dead PI3K-C2α did not display alteration of insulin signalling in insulin target tissues (Alliouachene et al. 2016). Although it cannot be excluded that the different results are due to the partial inactivation of the enzyme as opposed to its downregulation, the potential contribution of PI3K-C2α to insulin signalling requires further investigation.

8.2 PI3K-C2β

Compared to PI3K-C2α, the cellular functions ascribed to PI3K-C2β are somehow more limited, although they seem to be more common between different cell types. For example, PI3K-C2β is involved in regulation of migration of several cell types,

from HEK293 (Domin et al. 2005) and human umbilical vein endothelial cells (Tibolla et al. 2013) to different types of cancer cells (Maffucci et al. 2005; Mavrommati et al. 2016; Chikh et al. 2016; Katso et al. 2006). Investigation of the kinase-dead mouse model revealed a role for PI3K-C2β in the regulation of insulin signalling pathways through regulation of endosomal trafficking, in particular of the insulin receptor (Alliouachene et al. 2015). PI3K-C2β has been also involved in negative regulation of mTORC1 during growth factor deprivation (Marat et al. 2017) and it has been proposed as a host factor for influenza virus entry (O'Hanlon et al. 2019).

8.3 PI3K-C2γ

Investigation of the mechanisms responsible for the defective metabolism detected in PI3K-C2γ knockout mice revealed a role for this enzyme in the regulation of glycogen synthase activity in the liver upon insulin stimulation (Braccini et al. 2015). Overall, very little is still known about the physiological roles of PI3K-C2γ, which remains the most obscure of the three class II PI3Ks.

8.4 Co-operative Roles?

While most studies indicate distinct functions for class II PI3Ks, evidence of the involvement of both PI3K-C2α and PI3K-C2β in the regulation of some cellular processes has also appeared in the literature. Examples include studies indicating a role for both enzymes in vascular smooth muscle cell contraction (Sarker et al. 2019; Islam et al. 2020), in clathrin-dependent pinocytosis (Aung et al. 2019) and in the regulation of cell mitosis (Gulluni et al. 2017; Cisse et al. 2019). Understanding the relative contribution of each enzyme in these processes will be very interesting. It is known, for example, that they play distinct roles in the regulation of pinocytosis (Aung et al. 2019). On the other hand, the exact role of PI3K-C2β during mitosis has not been described yet (Cisse et al. 2019), therefore its involvement in this process is still not clear, in particular, compared to PI3K-C2α, which is critical for mitotic spindle assembly and anaphase onset (Gulluni et al. 2017). Interestingly, it has been demonstrated that the enzymatic activity of PI3K-C2α is not required during mitosis as it contributes to the process by acting as a scaffold protein (Gulluni et al. 2017). As the involvement of PI3K-C2β was identified using siRNA/shRNA-mediated downregulation (Cisse et al. 2019), it remains to be established whether the enzymatic activity of PI3K-C2β is required instead. In general, it will be interesting to define the exact mechanisms of action of each enzyme in cellular processes that involve both of them and to understand why two members of the same class of PI3Ks are required.

9 Class II PI3Ks, Immune Cells and Inflammatory Responses

Very few data suggesting a possible role for class II PI3Ks in immune cells are available and some of them are summarised here. Evidence includes the observation that downregulation of PI3K-C2α reduced the FcεRI-mediated release of the enzyme β-hexosaminidase and a tagged neuropeptide-Y in RBL-2H3 cells (Nigorikawa et al. 2014). These data, together with the detected localisation of PI3K-C2α on large vesicles generated upon FcεRI stimulation, suggested that this enzyme is involved in FcεRI-mediated degranulation (Nigorikawa et al. 2014).

A role for PI3K-C2β in the activation of the K^+ channel KCa3.1 was first suggested by the observation that siRNA-mediated downregulation of the enzyme inhibited the channel activity in Jurkat cells overexpressing KCa3.1 and in naïve human $CD4^+$ T cells (Srivastava et al. 2009). A specific role for PtdIns3*P* was also reported (Srivastava et al. 2009), consistent with previous studies (Srivastava et al. 2005, 2006a, b). In addition, reduced FcεRI-mediated KCa3.1 activation was detected in bone marrow-derived mast cells from PI3K-C2β knockout mice and this was rescued by dialysing a PtdIns3*P* able to insert into the plasma membrane (Srivastava et al. 2017). Interestingly, stimulation with anti-CD3 antibodies did not appear to increase PI3K-C2β enzymatic activity in Jurkat cells overexpressing both KCa3.1 and a GFP-tagged PI3K-C2β, but it appeared to recruit the enzyme to the immunological synapse, suggesting that relocation of the enzyme was crucial to impact on KCa3.1 (Srivastava et al. 2009). Additional analysis confirmed an important role for this PI3K-C2β/PtdIns3*P*/KCa3.1 pathway in mast cell activation, as bone marrow-derived mast cells from PI3K-C2β knockout mice displayed reduced Ca^{2+} influx, cytokine production and degranulation upon FcεRI stimulation (Srivastava et al. 2017). The additional observation that PI3K-C2β knockout mice were resistant to IgE-mediated passive cutaneous and systemic anaphylaxis led the authors to propose this enzyme as a potential therapeutic target for IgE-mediated diseases (Srivastava et al. 2017).

Involvement of class II PI3Ks in inflammatory responses in pathological conditions are also suggested by some data in the literature, although the specific contribution of these enzymes is not always defined. For instance, a recent study reported increased expression of PI3K-C2γ in the synovial fluid of rheumatoid arthritis (RA) compared to osteoarthritis (OA) patients, in cells of the synovial lining layers and inflammatory infiltrates in the synovial tissue of RA compared to OA patients and in peripheral blood mononuclear cells from RA patients compared to healthy individuals (Kim et al. 2020). These authors further demonstrated that the chemical compound PBT-6, which they showed inhibits PI3K-C2γ, was able to increase cell death in tumour necrosis factor (TNF)-α-induced synovial fibroblasts (MH7A cells) and in lipopolysaccharide (LPS)-activated Raw 264.7 macrophages. Furthermore, PBT-6 decreased levels of interleukin (IL)-6 in TNF-α-treated MH7A cells and secretion of TNF-α and IL-6 by LPS-activated Raw 264.7. Migration of activated macrophages towards the conditioned medium of TNF-α-induced fibroblasts was

also inhibited by addition of PBT-6. Importantly, the authors showed that PBT-6 inhibited osteoclastogenesis in vitro and reduced severity of collagen-induced arthritis in mice (Kim et al. 2020). On the other hand, a potential role for PI3K-C2α in OA has been suggested by a recent study reporting regulation of this enzyme by the potassium voltage-gated channel subfamily Q member 1 overlapping transcript 1 in chondrocytes (Liu et al. 2021). Specifically, the authors detected down-regulation of the channel in osteoarthritic compared to normal chondrocytes and showed that overexpression of PI3K-C2α reduced the secretion of inflammatory factors, reduced apoptosis and increased viability of osteoarthritic chondrocytes. These observations suggest that deregulation of this pathway might be involved in development or progression of OA (Liu et al. 2021).

A few observations suggesting a possible involvement of PI3K-C2β in the immune response in some pathological conditions also exist. Examples include a recent study evaluating the levels of nine tumour-infiltrating T cell types in hepatocellular carcinoma (HCC) and non-tumour tissues (Li et al. 2020). These authors suggested that driver genes including *PIK3C2B* might be associated with the reduced T cell infiltration detected in HCC (Li et al. 2020). Similarly, *PIK3C2B* has been found to be one of only two genes whose expression levels seem to be able to discriminate chronic lymphocytic leukaemia and monoclonal B cell lymphocytosis cases from normal polyclonal and mono/oligoclonal B lymphocytes (McCarthy et al. 2015).

Additional evidence in the literature still requires further investigation in terms of their significance. An example includes the observation that the antioxidant delphinidin, which was shown to ameliorate psoriasis in vitro and in vivo, was also reported to bind to some kinases, including PI3K-C2β (Chamcheu et al. 2017). The importance of such an interaction and whether it is associated with the detected beneficial effects was not clarified.

Whether future studies will reveal additional roles for class II PI3Ks in immune response in physiological and/or pathological conditions remains to be established. In this respect, it is worth mentioning that a pool of PtdIns3*P*, whose synthesis is mediated by class III PI3K, is produced in phagosomes and is important for phagocytosis (Vieira et al. 2001; Birkeland and Stenmark 2004; Ellson et al. 2001a), in particular for modulation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and superoxide production (Suh et al. 2006; Ellson et al. 2001b). In fact, the existence of cyclical waves of PtdIns3*P* synthesis during this process has been also reported (Chua and Deretic 2004). Although the potential involvement of class II PI3Ks in modulating PtdIns3*P* levels in this process was excluded by a study investigating the regulation of the NADPH oxidase during phagocytosis of *Staphylococcus aureus* and *Escherichia coli* (Anderson et al. 2008), whether additional studies will provide further information on this topic remains to be seen.

Similarly, it has been reported that autophagy is important for the regulation of the innate immune system (Germic et al. 2019a, b). Indeed, autophagy, a process generally used to degrade cellular components, such as damaged organelles or unnecessary and/or potentially harmful molecules, or to provide metabolic intermediates, can also provide materials for presentation by innate immune cells (Germic et al. 2019a). The process requires generation of a double membrane structure named

autophagosome and its fusion to lysosomes (Levine and Klionsky 2004; Zhao et al. 2021) and several studies have established that synthesis of PtdIns3*P* by class III PI3K is critical for autophagosome formation (Nascimbeni et al. 2017b; Backer 2008). Emerging data in the literature, however, also point to the contribution of PI3K-C2α to the process (Merrill et al. 2017), including recent evidence of a specific type of autophagy involving this enzyme (Bischoff et al. 2021). It will be interesting to investigate whether class II PI3K-mediated autophagy plays any role in immune cells.

10 Conclusion

Interest towards the identification of the physiological roles of class II PI3Ks and their potential involvement in human diseases has massively increased in the past fifteen years. This has led to a better understanding of the cellular functions regulated by these enzymes. On the other hand, some of the limitations that had previously hindered the investigation of class II PI3Ks still exist, namely the limited availability or lack of selectively inhibitors (Falasca et al. 2017), which has also strongly limited the impact and translational relevance of the accumulating lines of evidence pointing to their involvement in human diseases (Falasca et al. 2017). Much work is still required to shed more light into these PI3Ks and to fully understand their contribution to physiological and pathological processes.

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Class III PI3K Biology

Manuella Caux, Gaetan Chicanne, and Sonia Severin

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Abstract Highly conserved from yeast to mammals, vacuolar protein sorting 34 (Vps34) is the sole member of the third class of the phosphoinositide 3-kinase (PI3K) family. By producing phosphatidylinositol-3-monophosphate (PtdIns3P) through its scaffolding function essential for the catalytic lipid activity, Vps34 regulates endosomal trafficking, autophagy, phagocytosis, and nutrient-sensing signaling. The development of genetically modified mouse models and specific inhibitors has largely contributed over the past ten years to a better understanding of Vps34 functions in biological and physiological processes in mammals and, ultimately, its potential implications and targeting in human diseases. This chapter will summarize the current knowledge of the structure and regulation of Vps34 as well as its cellular and organismal functions.

Abbreviations

ALR	Autophagosome–Lysosome Reformation
AMPK	5′ Adenosine MonoPhosphate-activated protein Kinase
Atg14	Autophagy-related 14

M. Caux · G. Chicanne · S. Severin (✉)
Inserm U1297 and Paul Sabatier University, Institute of Cardiovascular and Metabolic Diseases, I2MC, 31432 Toulouse, France
e-mail: sonia.severin@inserm.fr

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ATP	Adenosine TriPhosphate
BARA	β-α Repeated Autophagy
BATS	Barkor/Atg14(L) autophagosome Targeting Sequence
BH3	Bcl-2 Homology 3
Bif1	Bax-interacting factor 1
CD46	Cluster of Differentiation 46
Cdk1	Cyclin-dependent-kinase 1
CHMP4	CHarged Multivesicular body Protein 4B
CRP	Collagen Related Peptide
DFCP1	Double-FYVE-domain-Containing Protein 1
EAE	Experimental Autoimmune Encephalomyelitis
EEA1	Early Endosomal Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ESCRT	Endosomal Sorting Complex Required for Transport
FBXL20	F-BoX and Leucine-rich repeat protein 20
FYVE-CENT	FYVE domain-containing CENTrosomal protein
$GABA_AR$	Gamma-AminoButyric Acid type A Receptors
GAP	GTPase-Activating Protein
GCPR	G-Protein Coupled Receptor
GEF	Guanine-nucleotide-Exchange Factor
GOPC	GOlgi-associated PDZ- and coiled-coil motif-containing
GTPase	GuanosineTriPhosphatase
HEAT	Huntingtin, Elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor
Hsp70	Heat-shock protein 70
HRS	Hepatocyte growth factor-Regulated tyrosine kinase Substrate
IGF1	Insulin-like Growth-Factor 1
IL	InterLeukin
KAP1	Kruppel-Associated Box domain-Associated Protein 1
KIF	KInesin Family member
LC3	Microtubule-associated proteins 1A/1B Light Chain 3B
mTORC1	Mammalian Target Of Rapamycin Complex 1
MHC	Major Histocompatibility Complex
MVB	MultiVesicular Bodies
NADPH	Nicotinamide Adenine Dinucleotide PHosphate
Nedd4	Neural-precursor-cell-expressed developmentally down-regulated 4
NK	Natural Killer
NRB1	NeuRaBin 1
NRBF2	Nuclear Receptor Binding Factor 2
PAQR3	Progestin and AdipoQ Receptor Family Member 3
PDK1	Phosphoinositide-Dependent protein Kinase 1
PGK1	PhosphoGlycerate Kinase 1
PKB	Protein Kinase B

PLD1	Phospholipase D1
PI3K	PhosphoInositide 3-Kinase
PIKFyve	Phosphoinositide Kinase, FYVE-type zinc finger containing
PtdIns3P	PhosphatidylInositol 3 monoPhosphate
PtdIns(3,5)P_2	PhosphatidylInositol 3,5-bisPhosphate
PX	PhoX
RACK1	Receptor for Activated C Kinase 1
Rubicon	Run domain protein Beclin 1-interacting and cysteine-rich containing
SGK3	Serum and Glucocorticoid Kinase 3
Skp1	Associated S-phase kinase-associated protein-1
SLAM	Signaling Lymphocyte-Activation Molecule
SNX	Sorting NeXins
TNF	Tumor Necrosis Factor
TRAF6	TNF Receptor-Associated Factor 6
TTC19	TetraTriCopeptide repeat protein 19
ULK1	Unc-51 Like autophagy activating Kinase 1
USP	Ubiquitin Specific Peptidase
UVRAG	UltraViolent irradiation Resistance-Associated Gene
Vps34	Vacuolar protein sorting 34
WIPI	WD-40 repeat protein Interactions with PhosphoInositides

Vacuolar protein sorting (Vps) 34 is the sole member of the third class of Phosphoinositide 3-Kinase (PI3K) family. Vps34 was first identified and characterized in *Saccharomyces cerevisiae* in the early 1990s by Emr laboratory (Herman and Emr 1990). Highly conserved from yeast to eukaryotic cells, Vps34 has been the focus of several laboratories over the past ten years for a better understanding of its structure, regulation, and role in mammals.

1 Vps34 Structure and Complexes

Vps34 is a 100 kDa ubiquitous protein structurally composed of an uncharacterized 52–54 amino acid N-terminal region, a C2 domain, a helical domain, and a C-terminal kinase domain, with the two latter domains being essential for Vps34 catalytic lipid activity (Backer 2008). Vps34-Vps15 forms a minimal dimeric membrane core complex essential for Vps34 catalytic activity (Herman et al. 1992; Volinia et al. 1995; Yan et al. 2009). Vps15, cloned in yeast by Emr laboratory (Herman et al. 1991) and conserved in mammals (Panaretou et al. 1997; Volinia et al. 1995), is a 150 kDa ubiquitous protein that consists of a N-terminal myristoylated pseudokinase domain followed by a central region containing Huntingtin, Elongation factor 3, the

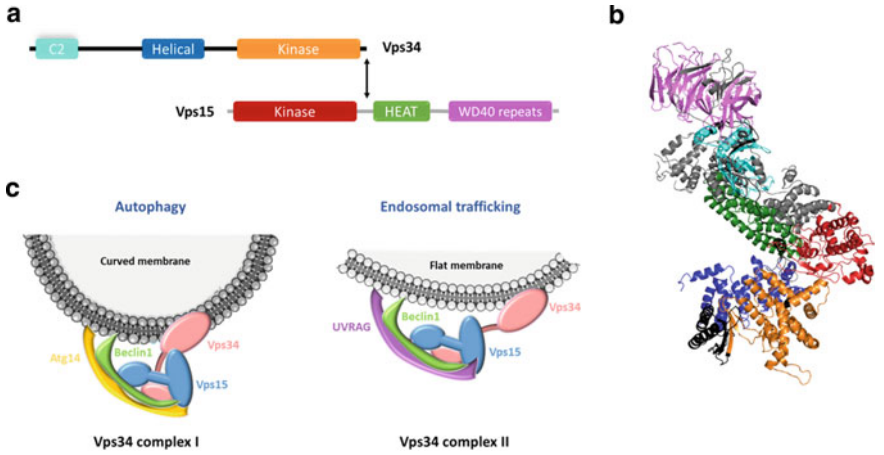

Fig. 1 **a** Structure of Vps34 and Vps15 forming the dimeric core complex. Vps34 is composed of a C2 domain (light blue), a helical domain (deep blue), and a C-terminal kinase domain (orange). Vps15 is composed of an N-terminal myristoylated pseudokinase domain (red) followed by a central region containing HEAT repeats (green) and a series of C-terminal WD-40 domains (purple). **b** Crystal structure of the Vps34-Vps15 complex in *Saccharomyces cerevisiae*. Vps34 and Vps15 domains are colored as in A. **c** Assembly and role of the Vps34 complexes I and II

PR65/A subunit of protein phosphatase 2A and the lipid kinase Tor (HEAT) repeats, and a series of C-terminal WD-40 domains (also known as beta-transducin) repeats that are short 40 amino acid domains often terminating in a Trp-Asp (W-D) dipeptide that forms a β-propeller-like structure (Stack et al. 1993; Herman et al. 1992) (Fig. 1a). The association of the N-terminal pseudokinase domain of Vps15 with the C-terminal kinase domain of Vps34 (Stack et al. 1993; Herman et al. 1992; Budovskaya et al. 2002) allows Vps34 membrane targeting and the exposition of the ATP-binding site of Vps34 kinase domain by conformational change of its helical domain. Both processes are essential for the lipid kinase activity of Vps34 (Stjepanovic et al. 2017a, b) (Fig. 1b).

The Vps34-Vps15 core complex can form a second complex with a coiled-coil and Bcl-2 Homology 3 (BH3) domain protein called Beclin 1 (Sinha and Levine 2008; Xu and Qin 2019). Considered as the most abundant complex in mammalian cells, Vps34-Vps15-Beclin 1 can bind to either autophagy-related 14 (Atg14) or ultraviolet irradiation resistance-associated gene (UVRAG) to, respectively form the tetrameric complex I or complex II. Cryo-electron microscopy and crystal structure analyses revealed that these two tetrameric complexes have a Y-shape organization that is governed on one side by the Vps15-Vps34 dimer and on the other side by the coiled-coil domains of Beclin 1 associated with either Atg14 (for complex I) or UVRAG (for complex II) (Rostislavleva et al. 2015; Ohashi et al. 2019; Stjepanovic et al. 2017b; Baskaran et al. 2014; Young et al. 2019). The two-arm tips of these tetrameric complexes allow their contact with the cell membrane through Vps34 kinase domain

and Beclin 1 C-terminal β-α Repeated Autophagy-specific (BARA) domain associated to the Barkor/Atg14(L) autophagosome Targeting Sequence (BATS) domain for complex I, or to the UVRAG BARA2 domain for complex II. The kinase activity of complex I depends critically on the ATG14 BATS domain, whereas complex II relies on the Beclin 1 BARA domain. The activities of Vps34 complexes I and II are regulated by various membrane physicochemical parameters: lipid unsaturation, negative charge, and membrane curvature. Unsaturated lipids increased the activation of both complexes and high membrane curvature can compensate for the negative effect of high lipid saturation. Both complexes are also activated by negatively charged phosphatidylserine (Ohashi et al. 2021). The curvature-sensing amphipathic helix in the Atg14 BATS domain allows complex I to preferentially associate with the highly curved autophagic isolation membrane (Fan et al. 2011), conferring a critical role for Vps34 complex I in the formation and elongation of the nascent autophagosome (Itakura et al. 2008; Matsunaga et al. 2009; Zhong et al. 2009; Lamb et al. 2013; Parzych and Klionsky 2014; Ravanan et al. 2017; Brier et al. 2019; Hurley and Young 2017). Through the flexibility between the two arms, Vps34 complex II acts on low-curvature flat endosomal membranes (Rostislavleva et al. 2015) to control endocytic/endosomal trafficking and to promote autophagosome–late endosome/lysosome fusion (Parzych and Klionsky 2014; Ravanan et al. 2017; McKnight et al. 2014; Thoresen et al. 2010) (Fig. 1c).

2 Vps34 Activity and Regulation

By phosphorylating phosphatidylinositol (PtdIns) at the position 3 of its inositol ring, Vps34 produces phosphatidylinositol-3-monophosphate (PtdIns3P) (Fig. 2a). Present in small amounts in cells, PtdIns3P is mainly found in early endosomes and autophagosomes (Schink et al. 2016, 2013) where it allows the relocalization of proteins containing specific domains with a high affinity for this lipid as the FYVE domain (for Fab1, YOTB, Vac1, Early Endosomal Antigen 1 (EEA1) domain) and the PhoX (PX) domain. Vps34 participates in the maintenance of a basal level of PtdIns3P in a cell type-dependent manner. Vps34 accounts for 65% of the basal PtdIns3P production in mouse embryonic fibroblasts (Devereaux et al. 2013), 50% in skeletal muscles (Reifler et al. 2014), 30–40% in megakaryocytes, and 10% in platelets (Bellio et al. 2020; Valet et al. 2017). Vps34 also contributes to the production of different stimulation-dependent pools of PtdIns3P that are regulated by the cellular environment and the subsequent involved Vps34 complex. For instance, glucose or amino acid starvation enhances the production of a Vps34 complex I-dependent autophagic pool of PtdIns3P (Russell et al. 2013; Kim et al. 2013), whereas insulin stimulation increases Vps34 complex II activity to promote endocytic trafficking (Nemazanyy et al. 2015). In platelets, Vps34 activity increases under collagen related peptide (CRP) or thrombin stimulation to produce an inducible pool of PtdIns3P that is important for thrombosis (Valet et al. 2017). In retinal rods, Vps34 appears to be the main kinase for light-induced PtdIns3P production (He et al. 2016).

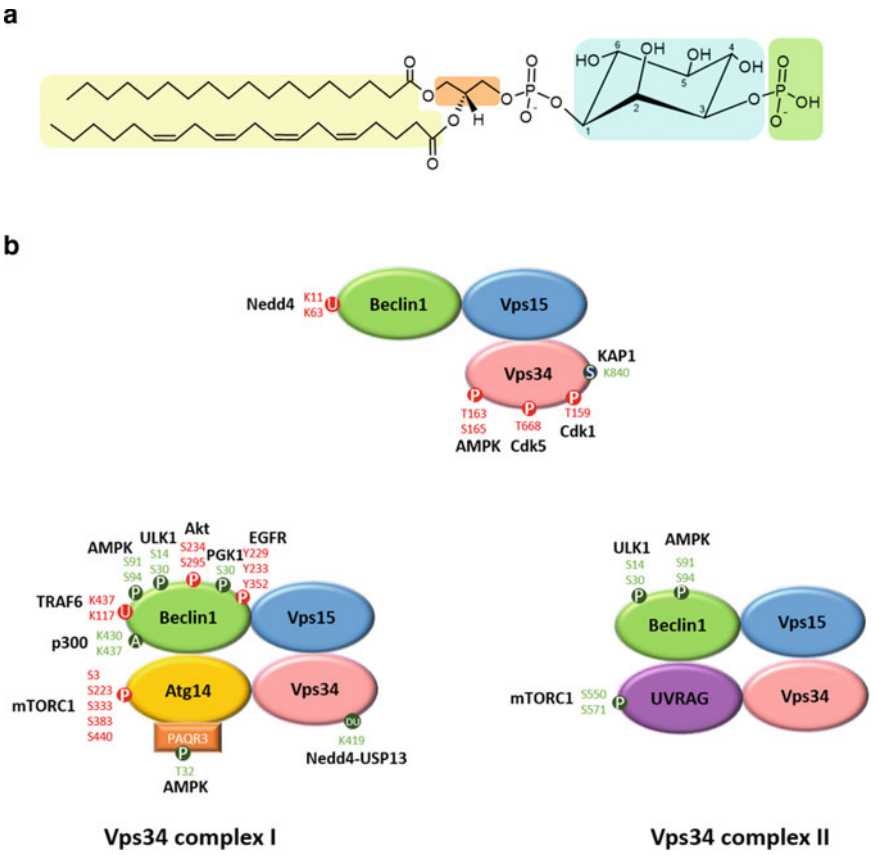

Fig. 2 **a** PtdIns3P structure. PtdIns3P is composed of a glycerol backbone (orange), which is connected by a phosphodiester bond to an inositol ring (blue) phosphorylated at its position 3 (green) in one side and to two fatty acids (yellow), predominantly stearic acid (C18:0) and arachidonic acid (C20:4) on the other side. **b** Regulation of Vps34 complexes by post-translational modifications. The members of Vps34 complexes can be positively (green) or negatively (red) regulated by different proteins by phosphorylation (P), sumoylation (S), acetylation (A), ubiquitinylation (U), or deubiquitinylation (DU)

To finely regulate Vps34 activity in response to specific cellular stimuli, Vps34 and the members of its different complexes (Beclin 1, Atg14, and UVRAG) can be subjected to post-translational modifications such as phosphorylation, sumoylation, acetylation, or ubiquitinylation (Backer 2016; Su and Liu 2018; Ohashi et al. 2019; Su et al. 2017; Menon and Dhamija 2018) (Fig. 2b). By phosphorylating Vps34 at threonine 163 and serine 165 residues on the C2 domain, 5′ adenosine monophosphate-activated protein kinase (AMPK) inhibits Vps34 lipid kinase activity of the non-autophagic Vps34 complexes (Byfield et al. 2005; Kim et al. 2013). AMPK can also indirectly regulate the lipid kinase activity of Vps34 present in complexes I and II by phosphorylating Beclin 1 at serine 91 and 94 residues under glucose deprivation (Kim

et al. 2013). In response to amino acid starvation, unc-51 like autophagy activating kinase 1 (ULK1) can also phosphorylate Beclin 1 at its serine 14 and 30 residues to initiate the Vps34 complex I-dependent autophagosome formation and enhance Vps34 complex I activity (Russell et al. 2013; Park et al. 2018). Phosphoglycerate kinase 1 (PGK1) also directly phosphorylates Beclin 1 at its serine 30 to enhance the autophagic activity of Vps34 activity in response to glutamine deprivation or hypoxia (Qian et al. 2017). AMPK also phosphorylates progestin and adipoQ receptor family member 3 (PAQR3) at its threonine 32 residue that is associated to Vps34 complex I under nutrient deprivation to induce autophagy (Xu et al. 2016). In contrast, Beclin 1 is also phosphorylated at serines 234 and 295 by AKT (also called protein kinase B [PKB]) leading to a decreased autophagy (Wang et al. 2012). In nutrient restoration conditions subsequently to the inhibition of AMPK and ULK1 pathways, mammalian Target Of Rapamycin Complex 1 (mTORC1) activation blocks the autophagic Vps34 complex I by phosphorylating Atg14 at serines 3, 223, 333, 383, and 440 (Yuan et al. 2013). Undergrowth factor stimulation (as Epidermal Growth Factor [EGF]), Beclin 1 is phosphorylated at tyrosines 229, 233, and 352, inducing a loss of Beclin 1 binding to Vps34 and a decreased Vps34 autophagic activity (Wei et al. 2013).

Vps34 can also be directly modified by sumoylation by kruppel-associated box domain-associated protein 1 (KAP1) on lysine 840 to increase Vps34 catalytic activity in vitro (Yang et al. 2013). Moreover, Beclin 1 is acetylated at lysines 430 and 437 by p300 acetyltransferase in order to promote the binding of run domain protein Beclin 1-interacting and cysteine-rich containing (Rubicon) to Beclin 1 and inhibit autophagosome maturation (Sun et al. 2015). Furthermore, expression and protein stability of Vps34 can be regulated through direct ubiquitinylation by Cul3-KLHL20 (Liu et al. 2016a) or by F-box and leucine-rich repeat protein 20 (FBXL20) and the associated S-phase kinase-associated protein-1(Skp1)–Cullin1 complex (Xiao et al. 2015). Vps34 degradation can also be promoted by its dissociation from its different complexes induced by various post-translational modifications. In fact, phosphorylation at threonine 159 residue by Cyclin-dependent-kinase 1 (Cdk1) and threonine 668 by Cdk5 inhibits Vps34-Beclin 1 interaction (Furuya et al. 2010). Moreover, Vps34 degradation is dependent of the ubiquitinylation of Beclin 1 at the lysine 117 and 437 residues by tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (Sun et al. 2015), of the cleavage of Beclin 1 by a caspase (Luo and Rubinsztein 2010), and of the modifications of the Beclin 1 expression that are dependent on ubiquitin ligases (neural-precursor-cell-expressed developmentally down-regulated 4 [Nedd4] (Platta et al. 2012) or Cul3-KLHL20 (Liu et al. 2016a)) and deubiquitinases (ubiquitin specific peptidase [USP] 10 and 13 (Liu et al. 2011)). Inversely, Nedd4-USP13 deubiquitination complex can stabilize Vps34 and promote autophagy by the cleavage of the polyubiquitin chains of Vps34 at the lysine 419 residue (Xie et al. 2020a, b). Scaffold/chaperon proteins interacting with Vps34 complexes can also regulate the formation and stability of Vps34 complexes and therefore Vps34 activity. This is the case for nuclear receptor binding factor 2 (NRBF2) (Cao et al. 2014; Lu et al. 2014; Young et al. 2016, 2019; Zhong et al. 2014), Ambra1 (Di Bartolomeo et al. 2010; Nazio et al. 2013; Fimia et al. 2007), receptor for activated C kinase 1 (RACK1) (Zhao et al. 2015; Xiao et al. 2018; Lee et al. 2019; Li et al. 2018)

and PAQR3 (Xu et al. 2016) for complex I, for Rubicon (Matsunaga et al. 2009; Zhong et al. 2009; Sun et al. 2011), Dapper1 (Ma et al. 2014) and Bax-interacting factor 1 (Bif1) (Takahashi et al. 2007, 2009) for complex II, and for heat-shock protein 70 (Hsp70) (Yang et al. 2013) or G-Protein Coupled Receptor (GCPR)-specific scaffold β-Arrestins (Wang et al. 2014) for Vps34-Vps15-Beclin 1 complex. Finally, changes in the assembly of Vps34 in its different complexes also modulate Vps34 activity. Vps34 activity is higher when the protein is present in complexes I or II, respectively associated with Atg14 or UVRAG, compared to its sole assembly to Vps15 in the dimeric core complex (Itakura et al. 2008).

3 Site of Actions and Cellular Roles of Vps34

Being present in different intracellular compartments, Vps34 displays a multitude of roles, with the predominant being the regulation of vesicular trafficking (Fig. 3). Vps34 is expressed in early endosomes and multivesicular bodies (MVB) to control endocytic/endosomal trafficking (Backer 2016; Bilanges et al. 2019; Raiborg et al. 2013). In early endosomes, Vps34 complex II binds to activated Rab5 through the WD-40 domain of Vps15 (Tremel et al. 2021; Buckles et al. 2020). The Vps34-dependent production of PtdIns3P synergizes with Rab5 in the recruitment of EEA1 and rabenosyn 5 to drive early endosomal tethering and fusion (Christoforidis et al. 1999a, b; Simonsen et al. 1998). In *Caenorhabditis elegans*, Vps34 inactivates Rab5

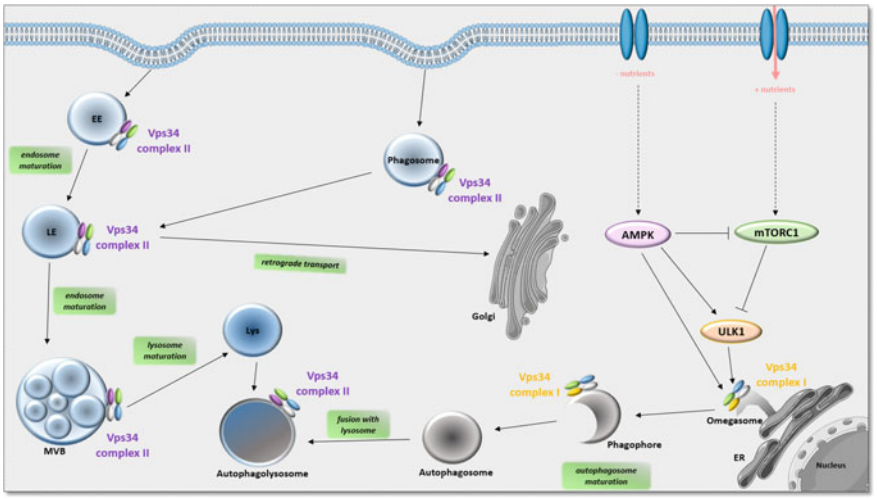

Fig. 3 Vps34 biological functions. (EE: Early Endosome, LE: Late Endosome, MVB: MultiVesicular Bodies, Lys: Lysosome, ER: Endoplasmic Reticulum, AMPK: 5'-Adenosine MonoPhosphate-activated protein Kinase, ULK1: Unc-51 Like autophagy activating Kinase 1, mTORC1: mammalian Target Of Rapamycin Complex 1)

via the recruitment of Tre-2, Bub2, and Cdc16 (TBC) -2 Rab guanosinetriphosphatase (GTPase) activating protein (GAP) to the Vps34-dependent pool of PtdIns3P to ensure the directionality of endosome maturation (Chotard et al. 2010; Law et al. 2017). Vps34, as an effector of Rab5, is also implicated in the movement of endosomes on microtubule tracks through the recruitment of kinesin family member (KIF) 16B, a PX domain-containing kinesin (Hoepfner et al. 2005; Nielsen et al. 1999). In MVB, a multiple protein complex the endosomal sorting complex required for transport (ESCRT) −0, −I, −II, and −III drives the internalization of ubiquitinylated membrane proteins into the intraluminal vesicles of these structures. PtdIns3P production by Vps34 at the endosome recruits the FYVE domain-containing ESCRT-0 protein, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), a key step for the subsequent assembly of additional ESCRT complex members essential for the intraluminal vesicle formation and the endosomal sorting of ubiquitinylated cargos. PtdIns3P also interacts with the GLUE domain (an atypical pleckstrin homology domain) of the ESCRT-II subunit to assist the endosomal targeting of ESCRT-II (Raiborg et al. 2001; Katzmann et al. 2003; Slagsvold et al. 2005; Teo et al. 2006). The PtdIns3P produced by Vps34 is also involved in the retrograde trafficking of endocytic cargos to the Golgi through the PX domain-containing sorting nxXins (SNX) 1, 2, 3, 5, and 6 (Dong et al. 2021; Harterink et al. 2011; Carlton et al. 2005; Cozier et al. 2002). In late endosomes, Vps34 complex II binds to Rab7 through the WD-40 domain of Vps15 and regulates Rab7 activation and late endocytic trafficking through recruitment of the GTPase-activating protein Armus (Stein et al. 2003; Jaber et al. 2016). In lysosomes, Vps34 inhibition affects procathepsin D maturation (Row et al. 2001). However, some effects of Vps34 inhibition on late endosome/lysosome homeostasis might be due to a decreased availability of PtdIns3P as a substrate for the phosphoInositide pinase FYVE-type zinc finger containing (PIKFyve) to produce phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P_2), both known to be implicated in the maintenance of late endosome and lysosome morphology and in the delivery of ubiquitinylated cargos to the lysosome (Shisheva 2012; Sharma et al. 2019; Dove et al. 2009). During phagocytosis mediated by receptors for opsonized particles, Vps34 complex II is recruited to the newly internalized phagosome by interacting with Rab5 and subsequently causes the phagosomal nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex binding to PtdIns3P that is essential for NADPH oxidase activation and reactive oxygen species production (Anderson et al. 2008; Kanai et al. 2001; Yang et al. 2012).

Vps34 is also required for both early and late steps of macroautophagy (Backer 2016; Bilanges et al. 2019; Nascimbeni et al. 2017), a physiological response to nutrient deprivation by which cells degrade their cytosolic content by forming a double-walled vesicular structure called autophagosome that eventually fuses with lysosomes (Ravanan et al. 2017; Lamb et al. 2013) (Fig. 3). Under nutrient deprivation, Vps34 present in complex I initiates autophagy by recruiting double-FYVE-domain-Containing protein 1 (DFCP1) and WD-40 repeat protein interactions with phosphoinositides (WIPI) 1/2 at the endoplasmic reticulum initial site of autophagosome formation called omegasome. Vps34 in complex II is involved in the final fusion of autophagosomes with lysosomes to form autophagolysosomes, but the

underlying mechanisms remain poorly characterized. After prolonged nutrient deprivation, Vps34 complex II also increases the number and length of lysosomal tubular lysosomes for the reformation of lysosomes in a process called autophagosome–lysosome reformation (ALR). This process involves mTORC1 by phosphorylating UVRAG at serine 550 and 571 residues (Kang et al. 2011; Xiao et al. 2018; Munson et al. 2015; Yu et al. 2010). Vps34 complex II is also directly recruited to the phagosome by binding to the signaling bacterial receptor signaling lymphocyte-activation molecule (SLAM) to increase autophagy (Berger et al. 2010; Dragovich and Mor 2018). Cluster of differentiation 46 (CD46), a receptor for measles virus, also binds to Vps34-Vps15-Beclin 1 complex via its associated scaffold protein GOlgi-associated PDZ- and coiled-coil motif-containing (GOPC) and activates autophagy (Joubert et al. 2009).

Vps34 can also regulate specific signaling pathways (Fig. 3). In the liver, Vps34 complex II activation downregulates insulin signaling by controlling insulin receptor trafficking (Nemazanyy et al. 2015). Conversely, Vps34 promotes epidermal growth factor receptor (EGFR) signaling by regulating the nuclear translocation of EGFR and the expression of $p14^{ARF}$ in non-small-lung-cancer cells (Row et al. 2001). In breast cancer cells, following growth factor stimulation (such as insulin-like growth-factor 1 [IGF1]), the serine/threonine kinase serum and glucocorticoid kinase 3 (SGK3) supplants AKT signaling for cell proliferation and survival (Vasudevan et al. 2009). SGK3 is recruited through its PX domain to the endosomal PtdIns3P produced by Vps34 complex II to be activated by the endosomal phosphoinositide-dependent protein kinase 1 (PDK1) and mTORC2 (Bago et al. 2014; Malik et al. 2018; Tessier and Woodgett 2006a, b). The AMPK pathway, a critical sensor of low cellular energy levels (reduced availability of key metabolites important for mitochondrial respiration and low levels of cellular Adenosine TriPhosphate (ATP)), is also controlled by Vps34 to induce autophagy (Bilanges et al. 2017). Paradoxically, in addition to its role in nutrient starvation-induced autophagy that in turn decreases mTORC1 signaling, Vps34 is also acting in nutrient (insulin or amino acid) supplementation conditions as a positive regulator of mTORC1 signaling (Dunlop and Tee 2014) that in turn inhibits Vps34 complex I by phosphorylating Atg14 (Yuan et al. 2013). Vps34-dependent translocation of phospholipase D1 (PLD1) to the lysosome appears to be critical for the nutrient-dependent regulation of mTORC1 (Yoon et al. 2011; Xu et al. 2011). Thus, Vps34, according to the specific complex that it is part of and to nutrient changes, plays specific but also contradictory roles.

Vps34 is also involved in specific cellular functions besides endosomal trafficking and autophagy. The components of Vps34 complex II (Vps34, Vps15, Beclin 1, UVRAG, and Bif1) were identified as novel regulators of cytokinesis (Thoresen et al. 2010). At the midbody, Vps34 present in complex II interacts with Gαi and the guanine-nucleotide-exchange factor (GEF) Ric-8A (Boularan et al. 2014) and produces PtdIns3P to stabilize the microtubule-dependent recruitment of FYVE domain-containing CENTrosomal protein (FYVE-CENT) and its binding partners, tetratriCopeptide repeat protein 19 (TTC19) and the associated microtubule motor KIF13A. The latter subsequently interacts with the ESCRT III protein, charged

multivesicular body protein 4B (CHMP4) involved in the abscission step of cytokinesis (Carlton and Martin-Serrano 2007). Vps34 also appears to play a role in the disposal of the midbody after abscission, which occurs via a selective macroautophagy process mediated by the autophagic receptor NeuRaBin 1 (NRB1) (Kuo et al. 2011). Moreover, Vps34 is important for the virulence of some pathogens as observed by the avirulence of Vps34-null *Cryptococcus neoformans* and *Candida albicans* strains (Hu et al. 2008; Jezewski et al. 2007) and the loss of *Salmonella* invasion in Vps34-invalidated host cells (Mallo et al. 2008).

4 Organismal Functions of Vps34

Through its main roles in endosomal trafficking and autophagy, Vps34 is important to maintain the homeostasis of different organs and cell types (Table 1) including neuronal systems (Zhou et al. 2010; Wang et al. 2011), heart (Jaber et al. 2012a, b; Kimura et al. 2017), liver (Jaber et al. 2012a, b; Bilanges et al. 2017), kidney (Grieco et al. 2018; Bechtel et al. 2013a, b), skeletal muscle (Bilanges et al. 2017; Reifler et al. 2014), megakaryocytes/platelets (Valet et al. 2017; Bertovic et al. 2020; Liu et al. 2017), retina (He et al. 2016, 2019; Rajala et al. 2020) and thyroid (Grieco et al. 2020). As total deletion of Vps34 in mice is lethal in early embryonic stages (between E7.5 and E8.5) (Zhou et al. 2011), studies deciphering the role of Vps34 in different tissues were allowed by the generation of mouse models with tissue-specific deletion of Vps34 using the Cre-Lox system or global heterozygous inactivation of Vps34 (Table 1).

There is increasing evidence that Vps34 plays several roles in the immune system. Using tissue-specific deletion of Vps34 mouse models, Vps34 has been shown to play a critical role in different populations of T lymphocytes (McLeod et al. 2011; Parekh et al. 2013; Willinger and Flavell 2012; Yang et al. 2021b), in dendritic cells (Parekh et al. 2017; Yang et al. 2021a) and in monocytes/macrophages (Yang et al. 2020) (Table 1). Vps34 also slows down the differentiation of myeloid progenitor cells to the macrophage lineage by regulating autophagy (Zhang et al. 2019). By regulating the microtubule-associated protein 1A/1B light chain 3B (LC3)-associated phagocytosis, Vps34 also prevents the monocyte/macrophage reprogramming to a pro-inflammatory phenotype in chronic liver diseases (Wan et al. 2020). In neutrophils, Vps34 is involved in phagosomal maturation as inhibition of Vps34 impaired phagosomal acidification and *S. aureus* destruction (Wood et al. 2020). By regulating the immune system, Vps34 is involved in cancer. The loss of Vps34 in monocytes/macrophages and dendritic cells promotes lung metastasis (Parekh et al. 2017). However, pharmacological inhibition of Vps34 limits melanoma and colorectal tumor growth by promoting infiltration of natural killer (NK) cells and T lymphocytes (Noman et al. 2020). Selective Vps34 inhibition appears to enhance the anti-proliferative activity and cytotoxic effect of the PI3Kδ inhibition against B cell malignant cells in the context of chronic lymphatic leukemia, indolent non-hodgkin lymphoma, and acute myeloid leukemia in vitro and in vivo (Liu et al. 2016b).

Table 1 Phenotype of different Vps34 mouse models

Tissues/Cell types	Mouse model	Phenotype	References
Neuronal system	*Advillin*-Cre *Pik3c3*$^{flox/flox}$ (sensory neurons)	Premature death (within 2 weeks after birth) Degeneration of mature sensory neurons Difficulties to coordinate movement and maintain body posture	Zhou et al. (2010)
	CaMKII-Cre *Pik3c3*$^{flox/flox}$ (pyramidal neurons)	Neurodegeneration Loss of dendritic spines Gliosis Potential massive atrophy of the entire cortex and hippocampus	Wang et al. (2011)
Heart	*Mck*-Cre *Pik3c3*$^{flox/flox}$	Cardiomegaly Decreased heart contractility with lower ejection fraction and fractional shortening Heart failure	Jaber et al. (2012a)
	Mck-Cre *Pik3c3*$^{flox/flox}$	Lethal hypertrophic cardiomyopathy-like disease Impaired cardiac proteostasis with destruction of myofibrils	Kimura et al. (2017)
Liver	*Alb*-Cre *Pik3c3*$^{flox/flox}$	Hepatomegaly Hepatic steatosis Increased liver protein content Lack of liver glycogen deposition	Jaber et al. (2012a)
	Vps34$^{D761A/+}$	Decreased gluconeogenesis Protection against hepatic steatosis Enhanced insulin sensitivity and glucose tolerance	Bilanges et al. (2017)
Kidney	*Pax8*-Cre *Pik3c3*$^{flox/flox}$	Fanconi-like syndrome: early kidney proximal tubular cell dysfunction (polyuria and low molecular weight proteinuria) Lethal kidney failure	Grieco et al. (2018)
	Nphs2-Cre *Pik3c3*$^{flox/flox}$	Impaired glomerular filtration (early proteinuria and glomerulosclerosis) Early lethality	Bechtel et al. (2013a)
Skeletal muscle	Vps34$^{D761A/+}$	Increased glucose uptake and glycolysis	Bilanges et al. (2017)

(continued)

Table 1 (continued)

Tissues/Cell types	Mouse model	Phenotype	References
	TgCkmm-Cre *Pik3c3*$^{flox/flox}$	Skeletal muscular dystrophy Severe dilated cardiomyopathy Progressive reduced mobility Early death (2 months after birth)	Reifler et al. (2014)
Retina	*Opsin*-Cre *Pik3c3*$^{flox/flox}$ (rod photoreceptors)	Progressive retinal degeneration with loss of rod electrical activity	He et al. (2016)
	Pcp2-Cre *Pik3c3*$^{flox/flox}$ (retinal secondary neurons)	Selective death of ON-bipolar cells Progressive decline of retina electrical activity	He et al. (2019)
	L/M-opsin-Cre *Pik3c3*$^{flox/flox}$ (cone photoreceptors)	Cone degeneration Loss of cone cell structure and function Increased death of cone cells	Rajala et al. (2020)
Thyroid	*Pax8*-Cre *Pik3c3*$^{flox/flox}$	Decreased thyroid hormonogenesis	Grieco et al. (2020)
Megakaryocytes/ Platelets	*Pf4*-Cre *Pik3c3*$^{lox/lox}$	Impaired platelet production (granule biogenesis and platelet size/count) Enhanced platelet secretion Defective thrombus formation Normal primary homeostasis	Valet et al. (2017)
	Pf4-Cre *Pik3c3*$^{flox/flox}$	Impaired aggregation and secretion Delayed clot retraction Defective thrombus formation Normal primary homeostasis	Liu et al. (2017)
	Pf4-Cre *Pik3c3*$^{flox/flox}$	Aberrant megakaryocytic maturation Decreased pro-platelet formation	Bertovic et al. (2020)
Monocytes/macrophages	*Lyz2*-Cre *Pik3c3*$^{flox/flox}$	Defective macrophage efferocytosis Reduced severity of EAE	Yang et al. (2020)
Dendritic cells	*Itgax*-Cre *Pik3c3*$^{flox/flox}$	Activated phenotype with cytokine production and increased MHC expression Defective homeostatic maintenance of splenic CD8α^{+} dendritic cells Defective cross-presentation of apoptotic cells to MHC class I-restricted T cells Increased metastasis growth	Parekh et al. (2017)

(continued)

Table 1 (continued)

Tissues/Cell types	Mouse model	Phenotype	References
	Itgax-Cre *Pik3c3*$^{flox/flox}$	Reduced severity of EAE Attenuated reactivation of primed T cells in the central nervous system	Yang et al. (2021a)
T lymphocytes	*Lck*-Cre *Pik3c3*$^{flox/flox}$	Impaired naive T lymphocyte homeostasis (increased cell death and reduced surface expression of InterLeukin (IL)-7Rα)	McLeod et al. (2011)
	Cd4-Cre *Pik3c3*$^{flox/flox}$	Impaired intrathymic development of natural killer T cells Global defect in regulatory T cells Inflammatory wasting syndrome: weight loss, intestinal inflammation, and anemia	Parekh et al. (2013)
	Cd4-Cre *Pik3c3*$^{flox/flox}$	Decreased naive T cell survival	Willinger and Flavell (2012)
	Cd4-Cre *Pik3c3*$^{flox/flox}$	Abortive differentiation of CD4 + T cells into T helper 1 cells Resistance to EAE	Yang et al. (2021b)

5 Vps34 in Human Diseases

The recent development of specific inhibitors of Vps34 (Bago et al. 2014; Dowdle et al. 2014; Pasquier et al. 2015; Ronan et al. 2014) opens exciting perspectives for a further characterization of Vps34 and its potential targeting in human pathology. For instance, one of the Vps34-specific inhibitors, SAR405, developed by Sanofi, can prevent memory consolidation by affecting autophagy and gamma-aminobutyric acid type A receptors ($GABA_AR$) trafficking with a potential therapeutic value for post-traumatic stress disorders (Li et al. 2019). To date, there are no recognized human diseases related to Vps34 mutations or expression levels. Only a microdeletion of the *PIK3C3* gene (encoding for Vps34) in one patient with a mental deficit and a deletion in exons 5–23 of *PIK3C3* gene in a patient with a learning deficit (Inaguma et al. 2016; Vulto-van Silfhout et al. 2013) have been described. Two studies also suggest a potential link between mutations in the *PIK3C3* gene promoter and psychiatric disorders such as schizophrenia and bipolar syndrome in humans (Stopkova et al. 2004; Tang et al. 2008).

6 Conclusions

Despite tremendous advances in Vps34 characterization in mammals, further studies are necessary to fill in the shadows on its regulation and its organismal role. The potential of Vps34 as a potential therapeutic target in specific pathological conditions remains an important question.

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PTEN in Immunity

Antonella Papa and Pier Paolo Pandolfi

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Abstract The tumor suppressor PTEN (Phosphatase and Tensin homolog deleted on Chromosome 10) executes critical biological functions that limit cellular growth and proliferation. PTEN inhibits activation of the proto-oncogenic PI3K pathway and is required during embryogenesis and to suppress tumor formation and cancer progression throughout life. The critical role that PTEN plays in restraining cellular growth has been validated through the generation of a number of animal models whereby PTEN inactivation invariably leads to tumor formation in a cell-autonomous fashion. However, the increasing understanding of the mechanisms through which the immune system contributes to suppressing tumor progression has highlighted how, in a cell non-autonomous fashion, cancer-associated mutations can indirectly enhance oncogenesis by evading immune cell recognition. Here, in light of the essential role of PTEN in the regulation of immune cell development and function, and

A. Papa (✉)
Cancer Program, Monash Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology, Monash University, Melbourne, VIC 3800, Australia
e-mail: antonella.papa@monash.edu

P. P. Pandolfi (✉)
Renown Cancer Institute, Nevada System of Higher Education, Reno, NV, USA
e-mail: PierPaolo.PandolfiDeRinaldis@renown.org

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based on recent findings showing that PTEN loss can promote resistance to immune checkpoint inhibitors in various tumor types, we re-evaluate our understanding of the mechanisms through which PTEN functions as a tumor suppressor and postulate that this task is achieved through a combination of cell autonomous and non-autonomous effects. We highlight some of the critical studies that have delineated the functional role of PTEN in immune cell development and blood malignancies and propose new strategies for the treatment of PTEN loss-driven diseases.

Abbreviations

ACT	Adoptive T-cell therapy
AKT	Protein Kinase B (Xie and Weiskirchen 2020)
AML	Acute myeloid leukemia
APC	Antigen-presenting cells
BCR	B cell receptors
BMDC	Bone marrow-derived dendritic cells
BTK	Bruton's tyrosine kinase
CAC	Colitis-associated colon cancer
CCL2	Chemokine (C–C motif) ligand 2
cDC	Conventional DCs
COVID-19	Coronavirus disease 2019
CSR	Class switch recombination
CTLA4	Cytotoxic T lymphocyte antigen 4
DCs	Dendritic cells
ERK1/2	Extracellular signal-regulated kinase 1/2
FDA	Food and Drug Administration
GBM	Glioblastoma multiforme
GPCRs	G-protein coupled receptors
HECT	Homologous to E6AP C-terminus
HSC	Hematopoietic stem cells
I3C	Indole-3-Carbinol
ICOS	Inducible T-cell COStimulator
IFN	Interferon
IGFR	Insulin-like Growth Factor Receptor
IL-12	Interleukin 12
IR	Insulin Receptor
IRF3	Interferon regulatory factor 3
ITK	Tyrosine-protein kinase ITK/TSK, a.k.a. interleukin-2-inducible T-cell kinase
LCK	Lymphocyte protein tyrosine kinase
LOH	Loss of heterozygosity
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase

MEFs	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
NEDD4	Neuronal precursor cell-expressed developmentally downregulated 4
NK cells	Natural killer cells
PD1	Programmed cell death protein 1
PDGFR	Platelet-Derived Growth Factor Receptor
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PH domain	Pleckstrin homology (PH) domain
PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphoinositide-3-kinases
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PTEN	Phosphatase and Tensin homolog deleted on Chromosome 10
RTKs	Receptor tyrosine kinases
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TAA	Tumor-associated antigens
T-ALL	T cell Acute Lymphoblastic Leukemia
TH1 cells	T helper 1 cells
TLR	Toll-like receptors
TNF	Tumor Necrosis Factor
Tregs	Regulatory T cells
TSC	Tuberous sclerosis complex
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
WWP1	WW Domain Containing E3 Ubiquitin Protein Ligase 1
WWP2	WW Domain Containing E3 Ubiquitin Protein Ligase 2

1 Introduction

Just over two decades ago, PTEN was identified as a putative tumor suppressor gene frequently deleted in advanced cancers of the prostate, brain, and breast (Li et al. 1997; Steck et al. 1997). In 1998, Maehama and Dixon and the Tonks lab reported that PTEN was the enzyme dephosphorylating the position 3 of the lipid substrate phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), and through this function, PTEN inhibited activation of the proto-oncogenic PI3K signaling pathway (Maehama and Dixon 1998; Myers et al. 1998).

Since its identification, the number of reports describing new PTEN alterations in cancer and in various neuronal developmental disorders has grown exponentially and to date, PTEN is known as one of the most frequently mutated and inactivated genes

in human diseases (Fruman et al. 2017; Lawrence et al. 2014). PTEN mutations can occur in somatic cancers and in the germline where they associate with the development of multiple cancer-predisposition syndromes known as PTEN Hamartoma Tumor Syndromes, PHTS (Papa and Pandolfi 2019; Yehia et al. 2019).

The essential role that PTEN plays in the maintenance of normal cellular and tissue homeostasis has been extensively characterized in multiple in vitro studies and through the generation of several animal models. Mouse models with complete PTEN loss, or complete loss of its lipid-phosphatase function, die early during embryogenesis demonstrating an essential role of PTEN in the regulation of embryonal development (Di Cristofano et al. 1998; Papa et al. 2014; Suzuki et al. 1998). Mice with PTEN heterozygous loss are instead viable and fertile, but undergo neoplastic transformation of epithelial cells, and develop multiple tumors (e.g., prostate, mammary gland, and endometrium), consistent with data observed in sporadic and heritable human cancers (Podsypanina et al. 1999; Stambolic et al. 2000).

Although more commonly observed in solid tumors, oncogenic PTEN inactivation also occurs in blood malignancies such as in T cell Acute Lymphoblastic Leukemia (T-ALL) (Gutierrez et al. 2009). In agreement with this, heterozygous PTEN deletion in the mouse causes lymphadenopathy and splenomegaly with a polyclonal expansion of B and T cells, succumbing by 12–13 months of age (Di Cristofano et al. 1998; Podsypanina et al. 1999; Suzuki et al. 1998). Conditional PTEN deletion in the hematopoietic stem cell (HSC) compartment or in the thymus also causes Acute Lymphoblastic Leukemia or T cell lymphoma, respectively, thus indicating that PTEN activity is essential for correct immune system development, maintenance, and function (Knobbe et al. 2008).

More recently, loss of PTEN has been also associated with resistance to a number of anti-cancer therapies including oncogene-targeted therapies as well as immune checkpoint blockade strategies which have revolutionized the clinical approach to treat cancer malignancies (Chen et al. 2019; Csolle et al. 2020; Peng et al. 2016; Yip and Papa 2021). Here, we summarize the multi-faceted functional role of PTEN in controlling immune cell development and function and provide an up-to-date overview of the consequences of loss of PTEN for cancer immunotherapies. We further discuss articles showing a critical role of PTEN in controlling viral innate immunity with a view to improve our understanding of biomarkers predicting response to ongoing and potential new therapies for infectious, autoimmune, and chronic inflammatory diseases, including COVID-19.

2 The Lipid and Protein Phosphatase PTEN: Many Functions, One Role

The best characterized mechanism through which PTEN acts as a tumor suppressor is through its lipid phosphatase function. PTEN directly dephosphorylates PIP3 and opposes the pro-survival signal induced by activation of the lipid kinase PI3K

(Fig. 1a). Physiologic accumulation of PIP3 at cell membranes recruits and activates a number of effector proteins including the kinase AKT which in turn amplifies the pro-growth signal by controlling the activation status of dozens of downstream targets including the mechanistic target of rapamycin, mTOR (Manning and Toker 2017). mTOR is a master regulator of cellular growth and can couple nutrient availability with mitogenic signals transduced by PI3K and AKT, and other oncogenic signaling pathways (e.g., Mitogen-Activated Protein Kinase, MAPK, pathway) (Liu and Sabatini 2020). Given the central role that PI3K pathway activation plays in supporting anabolic growth and proliferation, it is not surprising to find that many components of this cascade are frequently mutated in cancer. Cancer-associated

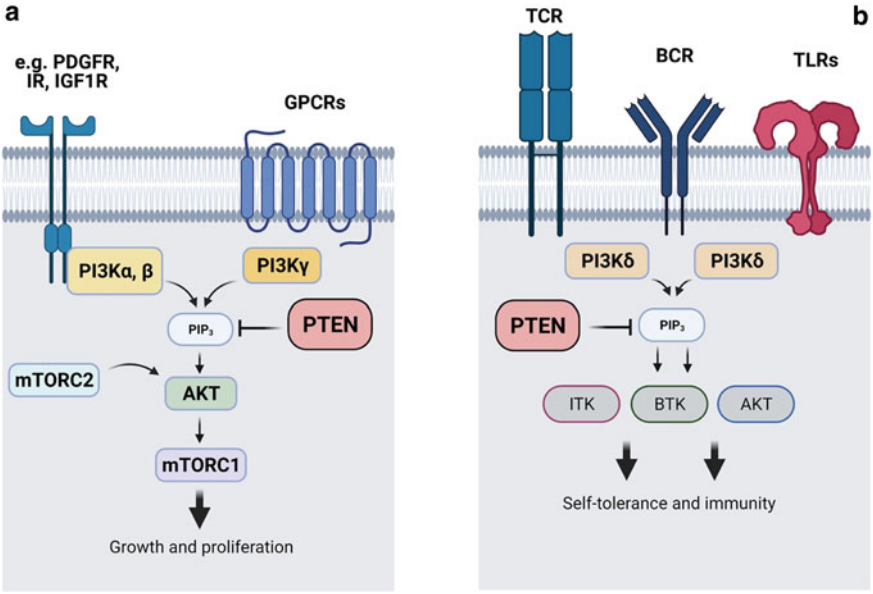

Fig. 1 PTEN inhibits PI3K signaling output in lymphoid and non-lymphoid cells. **a** Activation of receptor tyrosine kinases (e.g. Platelet-Derived Growth Factor Receptors, PDGFR, Insulin Receptor, IR, Insulin-like Growth Factor Receptor, IGFR) or G-Protein coupled Receptors (GPCRs) induce PI3K function and accumulation of the second messenger phosphatidylinositol-(3,4,5)-trisphosphate, PIP_3. Various PI3K isoforms and classes have been identified so far with PI3Kα being the most frequently mutated in cancer, and PI3Kδ more commonly expressed in immune cells (Fruman et al. 2017). PIP_3 accumulation activates AKT which in turn promotes mTORC1 functions and sustains cell growth and proliferation. mTORC2 phosphorylation of AKT promotes full AKT activation. **b** In immune cells, T cell receptors (TCR), B cell receptors (BCR), and Toll-like Receptors (TLRs) are activated in response to microbial infections. PI3Kδ activation generates PIP_3 which recruits and activates multiple proteins containing a pleckstrin homology (PH) domain including the Bruton's tyrosine kinase (BTK) and the inducible T cell kinase (ITK) (Yang et al. 2000). Activation of these non-receptor tyrosine kinases, in a coordinated manner, controls self-tolerance and promotes the innate and the adaptive immune response (Akira and Takeda 2004). By dephosphorylating PIP_3 in lymphoid and non-lymphoid immune cells, PTEN functions as a master regulator of multiple cellular processes.

mutations in *PIK3CA*, encoding the catalytic subunit of class IA PI3K, *PTEN, AKT,* and the tuberous sclerosis complex TSC, which inhibits mTORC1 functions, lead to constitutive activation of the pathway and frequently occur in breast, brain, prostate, and colon cancers (Fruman et al. 2017).

The repertoire of genetic alterations affecting PTEN function includes genomic deletion, silencing through promoter methylation or targeting of oncogenic microRNAs, as well as acquisition of nonsense and missense mutations (Lee et al. 2018). Although found along the entire gene sequence, PTEN point mutations cluster within the *exon 5*, encoding the PTEN catalytic domain (Bonneau and Longy 2000; Marsh et al. 1998). Of the several mutations occurring in the phosphatase domain, the PTEN G129E and PTEN C124S mutations are perhaps the most known and best characterized given their capacity to differentially affect PTEN catalytic activity. Indeed, in addition to the well-reported lipid phosphatase activity, PTEN was initially described as a dual-specificity protein phosphatase, and mutational screening showed that the cancer-associated PTEN G129E mutation could specifically affect PTEN lipid phosphatase function toward PIP3, while the PTEN C124S mutation was able to inhibit both lipid and protein phosphatase activity, thus rendering PTEN catalytically dead (Myers et al. 1997). Importantly, while over the years the role of PTEN lipid phosphatase function and inhibition of PI3K pathway activation has been strongly linked to tumor suppression, the contribution of PTEN protein phosphatase activity in the regulation of cellular growth has remained less defined despite the large number of reported proteins identified as direct PTEN substrates. Moreover, independent studies have highlighted how PTEN can still restrain cell proliferation even when devoid of its catalytic activity so that in a phosphatase-independent manner, PTEN maintains a degree of its tumor-suppressive activities (Chow and Salmena 2020; Lee et al. 2018; Papa 2016).

Collectively we conclude that irrespective of the modus operandi, the general consensus is that PTEN is a critical regulator of cell proliferation and cell survival and that it acts within cells to suppress growth, and in response to stressors to maintain cell integrity and preserve physiologic body functions.

3 PTEN Regulates Immune Cell Development and Function

PTEN and many components of the PI3K pathway are ubiquitously expressed in all cell types. PI3K pathway activation is induced by a number of upstream molecules such as receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCR) which receive activating signals from growth factors and hormones. PI3K also transduces the signal in response to activation of Toll-like receptors and B and T cell receptors and thus regulates the innate and adaptive immune responses (Fig. 1b) (Fruman et al. 2017; Vanhaesebroeck et al. 2010). The contribution of PI3K and its isoforms to regulation of immune cell function will be reviewed in separate chapters

within this book. Here, we will summarize the distinctive functional role of PTEN in controlling immune cell development as characterized by various laboratories that have studied mouse models with total body *Pten* heterozygous deletion, or with conditional PTEN knock-down driven by Cre recombinases induced by tissue, or lineage-specific promoters.

3.1 Germline PTEN Inactivation in Mice and Humans

Heterozygous *Pten* loss in mice ($Pten^{+/-}$ mice) leads to tumor development in multiple tissues with the most prominent phenotype being severe lymphadenopathy and splenomegaly occurring in both male and female mice. In the late '90s, three groups generated independent mouse lines with deletion of one *Pten* allele and they all similarly identified a pronounced lymphoid hyperplasia starting at ~5 months of age (Di Cristofano et al. 1998; Podsypanina et al. 1999; Suzuki et al. 1998). The lymphoid expansion involved the B and T cell compartments, was mostly polyclonal, and in a percentage of mice progressed to T cell leukemia/lymphoma, possibly due to loss of heterozygosity (LOH) of the remaining *Pten* wild-type allele (Suzuki et al. 1998). Some differences in phenotypes were observed in the percentage of apoptotic cells in various compartments. Popsypanina et al. observed reduced apoptosis in B cells and macrophages in the bone marrow, whereas Di Cristofano et al. found an impaired Fas-mediated apoptosis and increased proliferation of mature lymphocytes (Di Cristofano et al. 1999). The reduced sensitivity to induced cell death was found in B and T cells and in primary fibroblasts with heterozygous *Pten* loss compared to wild-type counterparts and was associated with an autoimmune phenotype (Di Cristofano et al. 1999).

Consistent with the central role of PTEN in regulating immune cell development, the generation of knock-in mice (KI) with heterozygous expression of the loss-of-function and PHTS-associated PTEN G129E mutation revealed that loss of PTEN lipid function recapitulated and exacerbated the phenotype observed in $Pten^{+/-}$ mice (Papa et al. 2014). More importantly, these findings also supported the notion that patients harboring germline PTEN mutations may be predisposed to develop immune dysfunctions and blood malignancies. Recently, case report studies and analyses on larger cohorts of PHTS patients with PTEN mutations have shown that a percentage of patients develop lymphoid hyperplasia and autoimmunity, though with milder severity compared to phenotypes observed in mouse models (Chen et al. 2017; Eissing et al. 2019).

Thus, heterozygous PTEN inactivation is sufficient to impair immune system development and this is dependent on the resulting activation of the PI3K pathway. However, the requirement for loss of heterozygosity (*loh)* at the *Pten* locus in $Pten^{+/-}$ mice diagnosed with leukemia/lymphoma, as described by Suzuki et al. (Suzuki et al. 1998), and the slow progression of the disease observed in PHTS patients, indicate that additional oncogenic mutations are needed to induce full neoplastic

transformation of leukocytes and that fail-safe mechanisms are in place to restrain disease progression upon loss of PTEN.

3.2 *Conditional* Pten *Deletion in Murine Models*

To address the functional role of PTEN in the regulation of immune cell development in a cell-autonomous fashion, numerous research groups have generated mouse models with conditional deletion of *Pten* at various stages of lymphocyte and myeloid cell development (Knobbe et al. 2008). These included analyses of mice with complete PTEN loss in the hematopoietic stem cells (HSC).

3.3 *Lymphocyte-Specific* Pten *Deletion*

In 2001, the Mak laboratory used the *Lck* (lymphocyte protein tyrosine kinase) promoter to enable thymocyte-specific *Pten* deletion in a *Pten* heterozygous background (*Lck-CrePten*$^{flox/-}$) (Suzuki et al. 2001). As expected based on the phenotype observed in *Pten*$^{+/-}$ mice, 6–8 weeks old *Lck-CrePten*$^{flox/-}$ mice already presented an increased number of thymocytes and splenocytes, and by 17 weeks of age, all experimental mice died of T cell lymphomas. *Lck-CrePten*$^{flox/-}$ -derived T cells proliferated faster, showed reduced apoptosis, and displayed higher levels of AKT and ERK1/2 activation relative to controls. Impaired negative thymic selection and an activated T cell signature were also identified consistent with the development of an autoimmune phenotype (Suzuki et al. 2001).

However, because this initial model carried a *Pten* heterozygous condition in the remaining body, whether partial loss of *Pten* in non-T cells contributed to the rapid onset of lymphomas and the autoimmune disease in *Lck-CrePten*$^{flox/-}$ mice was not defined. To this end, Hagenbeek et al. generated *Lck-CrePten*$^{flox/flox}$ mice first and *CD4-CrePten*$^{flox/flox}$ mice next, and demonstrated that except from some early thymic cellularity, complete *Pten* deletion at different stages of thymocyte development inevitably caused T cell lymphomas which originated in the thymus but could infiltrate other distant tissues, as previously reported (Hagenbeek et al. 2004; Hagenbeek and Spits 2008). The Turka lab further showed that the lymphoma developing in *Pten*-deficient CD4$^+$ T cells specifically depended on the loss of PTEN catalytic function (Newton et al. 2015). Moreover, analyses on *Pten*-deficient CD4$^+$ T cells, before full malignant transformation, showed that loss of PTEN also renders CD4$^+$ T cells hyper-responsive to TCR stimulation so that they no longer require co-stimulatory signals from CD28 or ICOS for full activation. Authors suggested that by controlling the levels of PI3K activation, the presence of PTEN defines a regulatory checkpoint during T cell activation which controls self-tolerance (Buckler et al. 2006).

Surprisingly, however, targeted *Pten* deletion in activated $CD4^+$ T cells (i.e., *OX40-CrePten*$^{flox/flox}$ mice) did not cause full malignancy in mice but only induced heightened cytokine production and increased lymphocyte counts in peripheral lymph nodes. These *Pten*-deficient T cells were defined as "superhelper" T cells because when transferred into recipient wild-type mice they more efficiently suppressed bacterial infections and growth of the EG7 thymoma cell line. The higher immune response achieved upon loss of PTEN led the authors to conclude that targeted PTEN deletion in mature T cells could be used as a strategy for cancer treatment through adoptive immunotherapies (Soond et al. 2012).

Given the central role of PTEN in controlling T cell development and self-tolerance, a few studies have also analyzed the consequences of *Pten* loss in the development and stability of regulatory T cells (Tregs) which are a specialized population of T cells that suppresses immune response and prevents autoimmunity. Shrestha et al. and Huynh et al. found that *Pten*$^{fl/fl}$*Foxp3*-Cre mice developed lymphoid hyperplasia with an expansion of unstable Tregs in the spleen and lymph nodes. Authors found that these unstable PTEN-deficient Tregs overtime lost expression of CD25 and Foxp3, exhibited lineage instability, and became effector-like T cells (Wan and Flavell 2007; Zhou et al. 2009). Mice showed increased numbers of T helper 1 (T_H1) and T follicular helper (T_{FH}) phenotypes which induced aberrant germinal B cell activation and disrupted humoral immunity (Huynh et al. 2015; Shrestha et al. 2015).

Although targeted *Pten* deletion in T lymphocytes can drive leukemia or lymphoma development, depending on the targeted T cell population, loss of *Pten* in B cell lymphocytes has been shown to only generate milder phenotypes. Two research groups independently analyzed B cell development in *CD19-CrePten*$^{flox/flox}$ mice and both reported increased counts of discrete B cell populations (B1 and Marginal Zone B cells), increased B cell proliferation in response to multiple stimuli, and impaired class switch recombination (CSR); however, B cell lymphomas were not observed. Authors concluded that because PTEN loss impaired isotype switching, which is essential for B cell lymphomagenesis, this prevented full oncogenic transformation of B cells (Shojaee et al. 2016; Suzuki et al. 2003).

Finally, loss of *Pten* also leads to an increased cellular turnover of natural killer (NK) cells. Based on analyses performed on transgenic mice expressing the Nkp46-driven Cre (i.e., Nkp46iCre) knock-in mice, authors found that PTEN loss caused a profound reduction of NK cell counts in spleen, liver, and lymph nodes but increased accumulation in the blood and mouse lungs. NK cells with loss of PTEN proliferated more but also died more than wild-type cells in vivo, and showed compromised compartmentalization since they more efficiently left the bone marrow and accumulated in peripheral blood (Leong et al. 2015).

Overall, the generation of many animal models with conditional PTEN deletion in various B and T cell populations supports the notion that PTEN is a critical regulator of multiple aspects of the lymphoid compartment which go from control of cell development to activation, from cell function to localization (Knobbe et al. 2008).

3.4 *Pten Loss in Myeloid Cells*

Genetic inactivation of PTEN in myeloid malignancies is not frequently observed, but post-translational modifications have been found to reduce PTEN function which associates with poor outcomes (Cheong et al. 2003; Morotti et al. 2015).

To study the role of PTEN in controlling myeloid cell development and innate immunity, multiple investigators have used the LysMcre transgenic model whereby the Cre recombinase is driven by the M lysozyme locus which causes gene deletion in granulocytes and macrophages (Clausen et al. 1999). Initial analyses by Zhu et al. found that bone marrow-derived *LysMcre-Pten*$^{-/-}$ neutrophils had extended survival in vitro and in vivo and that this depended on AKT activation (Zhu et al. 2006). Subramanian et al. further showed that *LysMcre-Pten*$^{-/-}$ neutrophils were also more sensitive to chemoattractants than wild-type cells, they presented a higher number of membrane ruffles, increased actin polymerization, and higher motility toward sites of infection (i.e., peritonitis models), thus indicating that PTEN should be considered as a target to increase neutrophil functions against sites of inflammation (Subramanian et al. 2007).

However, follow-up studies dissecting the function of *LysMcre-driven Pten* loss in macrophages in response to Leishmania infection found that although mice were eventually able to clear the pathogen through a normal production of T helper 1 (T_H1) cells, in the early phase of infections *Pten*$^{-/-}$ macrophages had reduced TNFα and IL-12 production and higher IL-10 levels than wild-type cells, thus indicating that PTEN expression is required for optimal innate immune activation in response to specific challenges (Kuroda et al. 2008).

Consistent with this, few studies have also analyzed the consequences of PTEN deletion specifically in dendritic cells (DCs) which, as many other immune cells, undergo a multi-step developmental process and depend on Flt3. In particular, authors found that *Itgax-Cre*-driven *Pten* deletion in DCs led to an expansion of conventional $CD8^+$ DCs (cDC) and $CD103^+$ cDCs in tissues, which relied on mTOR activation since treatments with the mTOR inhibitor rapamycin rescued the phenotype (Sathaliyawala et al. 2010). Importantly, the expansion of these specific DCs subtypes, while still capable of activating naïve T cells, was associated with a defective innate response to Listeria infection. The authors concluded that consistent with phenotypes observed in developing B cells, loss of PTEN impaired DC differentiation and was required to maintain proper ratios of peripheral cell subsets. Further analyses of conditional PTEN loss in similar mice confirmed the DC phenotype and also reported, a myeloproliferative disease and reduced neutrophil counts in the liver (Jiao et al. 2014).

Collectively, loss of PTEN in myeloid cells can cause defective development of various cell types across multiple lineages and can lead to impaired innate immune responses. This can have consequences for the management of infectious diseases as well as for tumor-immune surveillance, as we detail below.

3.5 *Pten Loss in HSC*

PTEN mutations occur in T-ALL and other hematological malignancies (Dahia et al. 1999; Gutierrez et al. 2009). With the goal of addressing the mechanisms underlying transformation of blood cells, and to identify cell populations responsive to PTEN loss, two groups used the interferon-induced *Mx1-Cre* system and deleted *Pten* in the hematopoietic stem cell compartment. Yilmaz et al. and Zhang et al. both reported that loss of PTEN led to rapid development of myeloproliferative disorders and extramedullary hematopoiesis, and within 4–6 weeks post-deletion, all PTEN-mutant mice progressed to leukemia (AML or ALL) and died (Yilmaz et al. 2006; Zhang et al. 2006). Remarkably, by studying the onset of the disease, the authors noticed that in addition to the leukemia phenotype, *Mx1-Cre-Pten*$^{-/-}$ mice showed a two–threefold expansion of HSCs compared to Cre-negative mice but surprisingly, these cycling HSCs were short-lived and if transplanted, they were no longer capable of long-term bone marrow reconstitution of irradiated recipient mice. Activation of mTOR played a key role in this process as rapamycin treatment restored the self-renewal capacity of *Pten*-deficient HSCs and also inhibited the generation as well as the maintenance of leukemia-initiating cells (Yilmaz et al. 2006). Critically, the authors concluded that while loss of PTEN can force HSCs to proliferate and expand, it also triggers a tumor suppressive response that exhausts the HSC pool so that only upon acquisition of additional oncogenic mutations, and bypassing of fail-safe mechanisms, *Pten*-deficient HSCs give rise to leukemia. In follow-up studies, crossing the *Mx1-Cre-Pten*$^{-/-}$ mice with additional oncogenic mutations, the Morrison lab showed that the tumor suppressive network activated upon loss of PTEN in HSCs relied on a few critical genes, e.g., p16 and p53, similar to findings in *Pten*-loss driven mouse models of prostate cancer (Chen et al. 2005; Lee et al. 2010). More recently, we have reported that fail-safe mechanisms driven by loss of PTEN also occur in breast cancer whereby inactivation of PTEN, through the loss of its protein phosphatase activity, promotes stability of the glucocorticoid receptor GR which induces death of PI3K-mutant mammary tumors and organoids (Yip et al. 2020). Further, Shojaee et al. unexpectedly found that retained PTEN expression contributed to the development of pre-B acute lymphoblastic leukemia (ALL). Authors reported that conditional loss of PTEN induced a condition reminiscent of autoreactive B cells and that by causing AKT hyper-activation and p53 function, PTEN loss induced cell death of pre-B ALL cells and prevented leukemia development (Shojaee et al. 2016).

Therefore, by targeting PTEN inactivation at different stages of immune cell development, and within distinct lineages of the immune system, we have learned that PTEN exerts multiple functions in response to specific cellular contexts, in turn leading to distinct outcomes. While in generic terms PTEN loss promotes cell growth and survival, it can also alter differentiation programs, or promote senescence and cell death if an excessive proliferative signal ensues (Fig. 2).

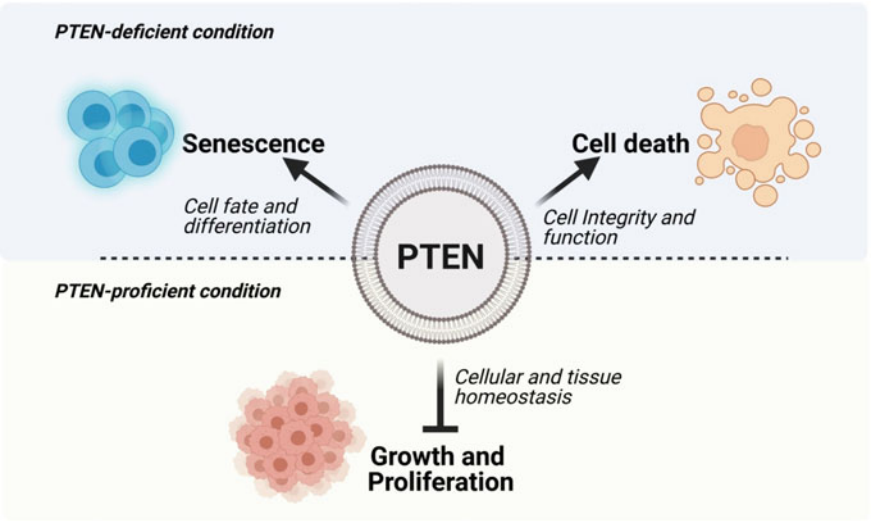

Fig. 2 PTEN dictates cellular outcomes. In response to growth stimuli, PTEN activation limits growth and proliferation, controls cellular differentiation, and induces death of dysfunctional cells. Loss of PTEN, which frequently occurs in cancer and other pathological conditions, sustains cell growth but also initiates fail-safe mechanisms that oppose excessive mitogenic signals by inducing senescence or cell death, in a context-dependent manner

4 PTEN Loss and Resistance to Immunotherapies

Cancer immunotherapy as a clinical treatment aims to enhance the capacity of a patient's adaptive immune response to fight neoplastic diseases. Whether based on the blockade of immune checkpoints, on adoptive T cell therapy (ACT), or on the delivery of monoclonal antibodies, the overall goal is to increase the T lymphocyte's ability to recognize tumor-associated antigens (TAA) and kill tumor cells (Waldman et al. 2020). Immune checkpoint therapy in particular has already contributed to improved outcomes and survival rates of patients affected by melanoma and lung cancer, and long-term cancer remissions have also been observed, thus making this therapeutic approach a new cornerstone for cancer treatment and cure (Ribas and Wolchok 2018).

The capacity of cytotoxic T cells to eliminate tumor cells depends on the recognition by T cell receptors (TCR) of tumor-specific antigens (neoantigens) presented by the major histocompatibility complex (MHC) on tumor cells. Full T cell activation requires a second co-stimulatory signal which is induced by binding of the CD28 receptor on T cells to its B7-1 and B7-2 ligands expressed on antigen-presenting cells (APCs), and tumor cells (Sharma and Allison 2015). The effect of these positive signals is however physiologically counter-balanced by the subsequent activation of negative immune regulators which prevent excessive T cell activation and proliferation, and also terminate the immune response once the pathogen, or the neoplasia, has

been cleared. Immune regulators such as cytotoxic T lymphocyte antigen 4 (CTLA4) oppose CD28 activation following antigen presentation by competing for the same ligands. Importantly, because CTLA4 recognizes B7-1 and B7-2 with greater affinity than CD28, CTLA4 induction leads to T cell anergy (Greenwald et al. 2001). Additional negative T cell regulators include the programmed cell death 1 (PD1) receptor which is also expressed on T cells after TCR stimulation and which, upon binding to its ligands PD-L1 and PD-L2 present on APCs, can induce T cell exhaustion (Waldman et al. 2020). Expression of PD-L1, also known as B7-H1 or CD274, can be found on many cell types in response to interferon-γ exposure released by activated T cells, and this event is seen as a mechanism used by normal cells to protect themselves from T cell attacks (Sharma and Allison 2015). Tumor cells use this same mechanism to escape T cell killing (Kalbasi and Ribas 2020; Waldman et al. 2020).

In immunotherapy, inhibition of CTLA4 and PD1, or PD-L1, through the administration of neutralizing antibodies, e.g., Ipilimumab and Nivolumab, can lead to prolonged activation of cytotoxic T cells and improved tumor clearance. Treatments with immune checkpoint inhibitors have generated better therapeutic responses than conventional therapies and have been approved by regulatory agencies (e.g., FDA) as standard treatments for melanoma, non-small cell lung cancer, and renal cancer (Ribas and Wolchok 2018).

Unfortunately, not all cancers have shown such positive response to immune checkpoint blockade as some melanoma patients did, and research into potential biomarkers of sensitivity or resistance is currently underway. Tumor mutational burden and levels of T cell infiltration in the tumor microenvironment are two of the most important biomarkers identified so far (Fridman et al. 2012; Lanitis et al. 2017; Rizvi et al. 2015).

Loss of *PTEN* can frequently occur in advanced stage cancers including brain cancer. In 2007, Parsa et al. found that PD-L1/B7-H1 levels increased post-transcriptionally upon loss of PTEN and that glioblastoma multiforme (GBM) samples with mutations or deletion of PTEN presented higher B7-H1 levels than samples retaining PTEN expression, thus initially linking the mutational status of PTEN to immuno-resistance (Parsa et al. 2007). In 2019, Zhao et al. performed genomic and transcriptomic analyses on human GBM samples resistant to the PD1 inhibitors pembrolizumab and nivolumab and confirmed a higher frequency of PTEN mutations in non-responder patients compared to responders. Loss of PTEN also prevented an increase in the recruitment of T cells to the tumor site, which was instead prominent in areas that retained wild-type PTEN (Zhao et al. 2019). Overall, PTEN inactivation correlated with an immunosuppressive microenvironment in brain cancer.

Consistent with these initial observations, Peng et al. found that loss of PTEN promoted resistance to immunotherapy in melanoma patients (Peng et al. 2016). By studying preclinical models and cohorts of melanoma patients resistant to anti-PD1 antibodies (pembrolizumab and nivolumab), the authors found that tumor areas with reduced PTEN expression showed a significant reduction of cytotoxic $CD8^+$ T cell

tumor infiltration. Loss of PTEN did not correlate with variations in PD-L1 expression, or in the levels of MHC class I molecules as previously reported, but significantly upregulated tumor CCL2 and VEGF expression and generated an immune-suppressive environment. In agreement with this, anti-VEGF blocking antibodies increased tumor infiltration and antitumor activity of transferred tumor-reactive T cells in xenograft models with PTEN-deficient melanoma cell lines (e.g., A375) (Peng et al. 2016).

Further, in trying to identify mechanisms of sensitivity to anti-PD1 checkpoint therapy, George et al. analyzed the primary tumor and the single metastasis of a patient diagnosed with uterine leiomyosarcoma who showed complete tumor remission for over 2 years after pembrolizumab monotherapy. Besides a significant decrease in the levels of $PD1^+$ T cell infiltration, and a reduction in neoantigens, loss of *PTEN* was identified as one of the few genetic events characterizing the resistant tumor (George et al. 2017).

Collectively, these initial studies all point to a critical role of PTEN in predicting response to immune checkpoint inhibitors and show that by inducing an immune-suppressive microenvironment, which limits cytotoxic T cell recruitment, selective loss of PTEN in tumor cells can affect tumor response to anti-PD1 antibodies (Fig. 3).

Importantly, PTEN can also affect tumor response to immunotherapy when its loss occurs in non-tumor cells, that is in myeloid cells. As described in Sect. 3.4, myeloid PTEN-deficiency can affect differentiation and function of dendritic cells and macrophages and, through PI3K pathway activation, this generates an M2-like state of "alternatively activated" macrophages (Gabrilovich et al. 2012). Whether these PTEN-deficient myeloid compartment can favor tumor growth was assessed by Kuttke et al. using the *LysMcre-Pten*$^{-/-}$ mice (*myPTEN*$^{-/-}$). Authors treated the *myPTEN*$^{-/-}$ mice with either azoxymethane (AOM) plus dextran sodium sulfate (DSS), to induce colitis-associated colon cancer (CAC), or subjected them to subcutaneous injections of the B16-F10 melanoma cell line. In both tumor models, PTEN-deficient myeloid cells favored bigger tumor development and mice showed a specific increase in splenic $CD8a^+$ DCs which failed to properly activate T cells, as supported by the fact that splenocyte cultures of *myPTEN*$^{-/-}$—$CD8^+$ T cells did not kill B16-Ova-Luc melanoma cells in vitro (Kuttke et al. 2016). Thus, this study demonstrates that PTEN can control tumor growth through mechanisms that impinge on cell-autonomous and cell non-autonomous effects. Given the occurrence of germline PTEN mutations in Cowden's Disease and other individuals affected by PHTS, we propose that an intrinsic increase in cellular growth, combined with a compromised tumor-immune surveillance, may both contribute to tumor upon formation germline loss of PTEN function.

5 PTEN and Viral Infections

Because PTEN influences the innate and adaptive immune responses against pathogens (Taylor et al. 2019), and given the particularly challenging times we live

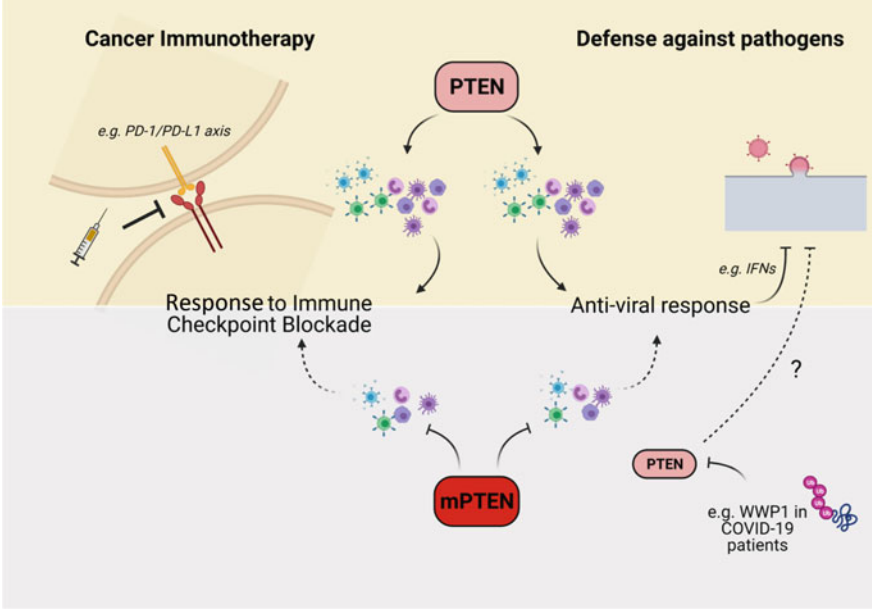

Fig. 3 PTEN regulates the immune system's response to various challenges. In cancer, PTEN expression associates with clinical response to cancer immunotherapy, in particular to inhibitors of the PD1-PD-L1 axis such as pembrolizumab and nivolumab. PTEN mutations or alterations (mPTEN) impair function of the myeloid compartment and generate an immunosuppressive tumor microenvironment which limits cytotoxic T cell activation and tumor infiltration. In turn, this causes resistance to immune checkpoint blockade strategies for cancer treatment. In response to pathogen infections, PTEN activates the myeloid and lymphoid responses. In particular, PTEN promotes interferons (IFNs) production to curb viral infections and mount an antiviral response. Consistently, loss of PTEN function impairs immune system activation and associates with worse outcomes in animal models. In COVID-19 patients, heightened expression of E3-ligases regulating PTEN levels and functions, such as WWP1, promotes viral egress and sustains viral infection. Whether COVID-19 patients also show reduced PTEN levels and function, and whether this further contributes to an exacerbation of the infection is not currently known

in due to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the associated coronavirus disease 2019 (COVID-19), we found important to also highlight reports describing a role for PTEN in the fight against viral infections.

Type I interferons are critical regulators of the antiviral response because they act in multiple ways by inhibiting viral replication and also by promoting the early as well as the adaptive immune response (McNab et al. 2015; Murira and Lamarre 2016). In 2015, Li et al. reported that PTEN regulates the nuclear import and consequent activation of interferon regulatory factor 3 (IRF3), a critical transcription factor controlling the expression of type I interferons (Li et al. 2016). Authors found that conditional PTEN loss induced by the *Ubc-CreER* caused a significant reduction of interferon-alpha (IFN-α) and beta (IFN-β) production following infection with the vesicular stomatitis virus (VSV). This in turn caused higher VSV titers and reduced

survival of PTEN-mutant mice compared to wild-type animals. Targeted PTEN deletion in mouse embryonic fibroblasts (MEFs) and bone marrow-derived dendritic cells (BMDCs) further confirmed that loss of PTEN impaired type I interferon production in vitro in response to various infections, including RNA and DNA viruses and lipopolysaccharide (LPS) (Li et al. 2016).

More recently, alterations in the expression levels and function of regulators of PTEN protein activation have been implicated in supporting viral replication, including viral exit from cells, i.e., cell egression of SARS-CoV-2 (Novelli et al. 2021). Specifically, higher mRNA and protein levels of members of the E3-ubiquitin ligase family such as WWP1, WWP2, and NEDD4 have been identified in human samples and cells infected with SARS-CoV-2 (Novelli et al. 2021). Moreover, a direct interaction between a few Homologous to E6AP C-terminus (HECT) family (HECT)-E3 ubiquitin-ligases and the SARS-CoV-2 spike protein was observed, accompanied by the ubiquitylation of the SARS-CoV-2 spike protein. WWP1, WWP2, and NEDD4 have all been reported to regulate PTEN activation in various ways (Lee et al. 2019; Maddika et al. 2011; Wang et al. 2007). In particular, WWP1 was shown to inhibit PTEN dimerization, and pharmacological WWP1 inhibition was able to reactivate PTEN function at the plasma membrane (Lee et al. 2019). WWP2 was instead reported to target PTEN for ubiquitin-dependent proteasome-mediated degradation (Maddika et al. 2011). Notably, germline variants of WWP1 and other HECT-E3-ligases have been identified in critically ill COVID-19 patients (Novelli et al. 2021) indicating the presence of a potential functional link between these selected germline variants and SARS-CoV-2 cell egression. Moreover, because WWP1 and WWP2 repress PTEN activation and protein levels, their increased expression in SARS-CoV-2 infected cells compared to control samples may in turn affect PTEN function and hence impair the patient immune responses. On the basis of these observations, whether PTEN levels and function are indeed altered in patients affected by COVID-19 is a topic worth investigating. Lastly, because WWP1 function can be inhibited with Indole-3-Carbinol (I3C), a well tolerated natural compound derived from cruciferous vegetables (Lee et al. 2019), this can pave the way to I3C clinical testing in COVID-19 and other viral infections.

6 Conclusions

Here we have provided an overview of the various roles that PTEN plays in controlling immune cell development and function and have included reports describing how PTEN acts as a critical regulator of the innate and adaptive immune response. We have discussed how through intrinsic and extrinsic mechanisms, loss of PTEN can affect tumor growth and also response to cancer immunotherapy, and have indicated that targeted inhibition of mTORC1 can rescue some of the phenotypes driven by conditional PTEN loss in murine models. Based on this, we suggest that treatments directed at critical components of the PI3K pathway, including PI3K and AKT inhibitors, can help correct some of the alterations caused by somatic and

germline PTEN mutations, including dysfunctions in the immune system. Moreover, in light of the critical role of PTEN in predicting response to immune checkpoint inhibitors such as pembrolizumab and nivolumab, we also suggest that a combination of oncogene-based targeted therapies, immunotherapy, and enhancement of PTEN activation through I3C administration, could provide a new opportunity to treat diseases associated with functional loss of PTEN (Fig. 3). This ever-evolving precision medicine approach can bring new hope to cancer patients and further treatments for infectious diseases.

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PHLPP Signaling in Immune Cells

Gema Lordén, Avery J. Lam, Megan K. Levings, and Alexandra C. Newton

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Abstract Pleckstrin homology domain leucine-rich repeat protein phosphatases (PHLPP) belong to the protein phosphatase magnesium/manganese-dependent family of Ser/Thr phosphatases. Their general role as tumor suppressors has been documented for over a decade. In recent years, accumulating evidence suggests that PHLPP isozymes have key regulatory roles in both innate and adaptive immunity. In macrophages, PHLPP1 dampens signaling through TLR4 and the IFN-γ receptor

G. Lordén and A. J. Lam: Contributed equally.

G. Lordén · A. C. Newton (✉)
Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093, USA
e-mail: anewton@health.ucsd.edu

A. J. Lam · M. K. Levings
Department of Surgery, University of British Columbia, Vancouver, BC V5Z 4H4, Canada

BC Children's Hospital Research Institute, Vancouver, BC V5Z 4H4, Canada

M. K. Levings
School of Biomedical Engineering, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

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by altering cytosolic signaling pathways. Additionally, nuclear-localized PHLPP1 inhibits STAT1-mediated inflammatory gene expression by direct dephosphorylation at Ser 727. PHLPP1 also regulates the migratory and inflammatory capacity of neutrophils in vivo. Furthermore, PHLPP1-mediated dephosphorylation of AKT on Ser 473 is required for both the suppressive function of regulatory T cells and for the pro-apoptotic effects of PHLPP1 in B cell chronic lymphocytic leukemia. In the context of immune homeostasis, PHLPP1 expression is modulated in multiple cell types by inflammatory signals, and the dynamics of its expression have varying effects on the pathogenesis of inflammatory bowel disease and septic shock. In this review, we summarize recent findings on the functions of PHLPP in inflammatory and regulatory signaling in the context of both innate and adaptive immunity.

Abbreviations

AGC	Related to protein kinase A, protein kinase G, protein kinase C
AP-1	Activator protein-1
BCR	B cell receptor
Cdk	Cyclin-dependent kinase
CLL	Chronic lymphocytic leukemia
CREB	CAMP-response element-binding protein
DAMPs	Damage-associated molecular patterns
DSS	Dextran sodium sulfate
EGFR	Epidermal growth factor receptor
Gbp5	Guanylate-binding protein 5
HDAC3	Histone deacetylase 3
IBD	Inflammatory bowel disease
IFN	Interferon
IFNGR	Interferon-γ receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF3	Interferon regulatory factor 3
IκB	Inhibitor of nuclear factor κB
KLA	Kdo2-lipid A
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAPKs	Mitogen-activated protein kinases
NES	Nuclear export sequence
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NTE	N-terminal extension
PAMPs	Pathogen-associated molecular patterns
PH	Pleckstrin homology
PHLPP	Pleckstrin homology domain leucine-rich repeat protein phosphatases

PHTS	PTEN hamartoma tumor syndrome
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PPM	Protein Ser/Thr phosphatase magnesium/manganese-dependent
PRRs	Pattern recognition receptors
RTK	Receptor tyrosine kinase
SP1	Specificity protein 1
STAT	Signal transducer and activator of transcription
Tconvs	Conventional T cells
TCR	T cell receptor
TGF	Transforming growth factor
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
Tregs	Regulatory T cells

1 Introduction

Phosphorylation plays a key role in the immune system, which comprises many biological processes an organism possesses to defend itself against foreign agents. The immune system consists of both innate and adaptive arms, with the former occurring in a rapid but non-specific manner, and the latter in a slower but more specific manner. Innate immune cells, such as macrophages, neutrophils, natural killer cells and dendritic cells, detect and eliminate invading pathogens or inappropriate damage by recognizing pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) via their pattern recognition receptors (PRRs) (Lu et al. 2008). Receptor engagement initiates signaling cascades that culminate in a variety of pathways related to host defense as well as the recruitment and activation of adaptive immune cells, namely B cells and T cells. The orchestration of an adaptive immune response relies on antigen recognition by B or T cell receptors (BCRs and TCRs) and has the potential to form immune memory. Engagement of BCRs and TCRs analogously triggers signaling cascades critically regulated by protein kinases and phosphatases.

Immune processes are tightly regulated at multiple levels, including epigenetic, transcriptional, post-transcriptional, translational, and post-translational (Liu et al. 2016). Among post-translational mechanisms, protein phosphorylation and dephosphorylation play important regulatory roles in both innate and adaptive immune responses (Liu et al. 2016). Aberrant signal transduction can lead to immune dysregulation and pathology. Understanding and modulating the basis of normal and pathological states of immune cells has thus become an area of particular interest. However, whereas much is known about kinases within these pathways, less is known about the phosphatases that regulate the immune response. An emerging player in the control of both innate and adaptive immune responses is the pleckstrin homology domain

leucine-rich repeat protein phosphatase (PHLPP) family, one of the most recently characterized members of the phosphatome (Gao et al. 2005; Brognard et al. 2007).

1.1 PHLPP Phosphatases: Structure and Function

PHLPP isozymes belong to the protein phosphatase magnesium/manganese-dependent (PPM) family of Ser/Thr phosphatases. Their catalytic activity depends on Mn^{2+}/Mg^{2+}, and they are insensitive to common phosphatase inhibitors such as microcystin and okadaic acid (Gao et al. 2005; Grzechnik and Newton 2016). Presently, the PHLPP family consists of two members, PHLPP1 and PHLPP2, each encoded by separate genes. PHLPP1 was first identified in 2005 following a rational search for an AKT-directed phosphatase (Gao et al. 2005); two years later, a second member of the PHLPP family was identified and named PHLPP2 (Brognard et al. 2007). Both isozymes of PHLPP are evolutionarily conserved from yeast to humans (Newton and Trotman 2014).

Unlike most other Ser/Thr phosphatases, PHLPP isozymes are multi-domain enzymes whose regulatory regions are located on the same polypeptide (Grzechnik and Newton 2016). Both PHLPP family members share a similar structure with several conserved domains including a pleckstrin homology (PH) domain, a hydrophobic leucine-rich repeat (LRR) region, a catalytic PP2C phosphatase domain, and a PDZ (post-synaptic density protein PSD95, Drosophila disk large tumor suppressor DLG1, and zonula occludens-1 protein zo-1)-binding motif (Gao et al. 2005; Grzechnik and Newton 2016). These regulatory domains are essential for appropriate intracellular targeting of PHLPP to access specific downstream substrates, crucial for its biological functions. The PH domain is required to dephosphorylate cellular protein kinase C (PKC) (Gao et al. 2008), the LRR region enables regulation of receptor tyrosine kinase (RTK) transcription (Reyes et al. 2014), and the C-terminal tail and PDZ-binding motif are important for scaffold binding and AKT dephosphorylation (Brognard et al. 2007) (Fig. 1). Because the catalytic efficiency of the phosphatase domain is relatively low (Sierecki and Newton 2014), coordination of this domain in close proximity to its substrates, mediated by the regulatory domains, is essential for PHLPP biology.

PHLPP1 differs from PHLPP2 in several aspects. Strikingly, PHLPP1 possesses a lengthy N-terminal extension (NTE) of approximately 50 kDa that is phosphorylated by cyclin-dependent kinase (Cdk) 1 during mitosis, regulating the protein interaction network of PHLPP1 (Cohen Katsenelson et al. 2019; Kawashima et al. 2021). This NTE contains a nuclear localization sequence (NLS) required for PHLPP1 nuclear translocation (Cohen Katsenelson et al. 2019). Although poorly conserved through evolution, the NTE is necessary for dephosphorylation of a recently identified substrate of PHLPP1, the signal transducer and activator of transcription (STAT) 1 (see Sect. 2.2). However, it is not required for dephosphorylation of other known substrates, including AKT (Gao et al. 2005; Brognard et al. 2007), PKC (Gao et al. 2008), and S6K1 (Liu et al. 2011). PHLPP1 also contains a nuclear export sequence

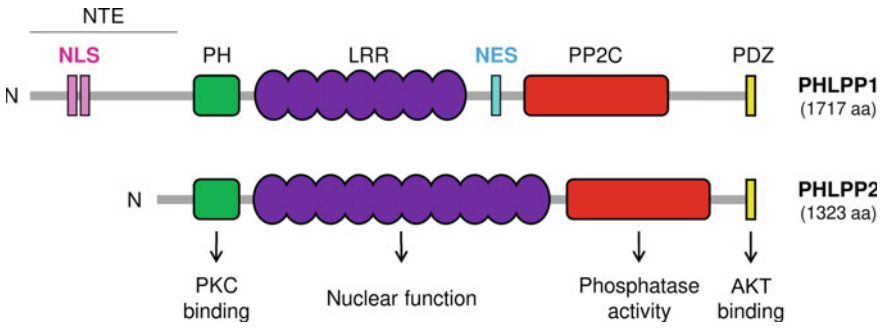

Fig. 1 PHLPP1 and PHLPP2 protein structure. The PHLPP family of phosphatases comprises two isozymes: PHLPP1 and PHLPP2. Both isozymes have similar domain structures, including a pleckstrin homology (PH) domain required for targeting to PKC, a leucine-rich repeat (LRR) required for nuclear PHLPP function, a PP2C phosphatase domain, and a PDZ-binding motif required for targeting AKT. Unlike PHLPP2, PHLPP1 also has a large, 50-kDa N-terminal extension (NTE) containing a nuclear localization signal (NLS), as well as a nuclear export sequence (NES) between the LRR and the PP2C phosphatase domains. Adapted from (Grzechnik and Newton 2016)

(NES) between the LRR region and PP2C domain that is not present in PHLPP2 (Cohen Katsenelson et al. 2019) (Fig. 1). Finally, both proteins diverge in their C-terminal sequences, which include the PDZ-binding motifs.

1.2 PHLPP Substrates

The first identified substrate of PHLPP is AKT, a member of the AGC (related to protein kinase A, protein kinase G, and protein kinase C) family of protein kinases, suggesting a role for PHLPP in opposing growth factor signaling (Gao et al. 2005). PHLPP directly and selectively dephosphorylates the hydrophobic motif of AKT (Ser 473 on AKT1), a key regulatory site, without affecting the activation loop (Thr 308 on AKT1). Although PHLPP1 and PHLPP2 both dephosphorylate the same residue on AKT, PHLPP isozymes differentially regulate AKT isozymes in cells: PHLPP1 dephosphorylates AKT2, whereas PHLPP2 dephosphorylates AKT1. Both isozymes dephosphorylate AKT3 (Brognard et al. 2007). Because full activation of AKT requires phosphorylation at both Ser 473 and Thr 308, PHLPP-mediated dephosphorylation of the AKT hydrophobic motif results in inactivation of the kinase. Thus, as terminators of phosphoinositide-3-kinase (PI3K)-AKT signaling, PHLPP proteins are considered tumor suppressors, limiting cell proliferation and tumor growth and promoting apoptosis (Fig. 2) (Gao et al. 2005; Brognard et al. 2007).

PHLPP is now known to dephosphorylate several other AGC kinases on their hydrophobic motifs, as well as other kinases on unrelated phosphorylation switches (Fig. 2) (Grzechnik and Newton 2016). Both PHLPP isozymes dephosphorylate

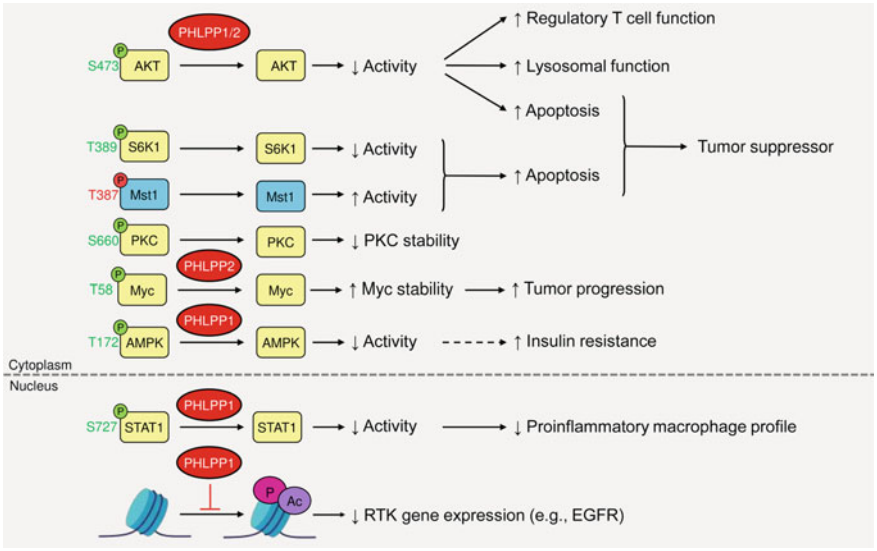

Fig. 2 PHLPP1 and PHLPP2 substrates and function. Both PHLPP1 and PHLPP2 can regulate several cellular pathways through different substrates. PHLPP1/2 directly dephosphorylate AGC kinases such as AKT, S6K1, and PKC at their hydrophobic motifs (green), resulting in kinase inactivation (AKT and S6K) or increased degradation and hence decreased kinase activity (PKC). PHLPP1/2 also dephosphorylate non-AGC kinases such as the pro-apoptotic kinase Mst1 at an inhibitory site (red). In addition, PHLPP2 dephosphorylates Myc (Thr 58) to promote its stability, and PHLPP1 dephosphorylates AMPK at its activation site (Thr 172). Finally, in the nucleus, PHLPP1 directly dephosphorylates STAT1 at Ser 727, and both PHLPP1 and PHLPP2 inhibit receptor tyrosine kinase (RTK) expression via suppression of histone acetylation and phosphorylation

the hydrophobic motif of PKC (Ser 660 on PKCβII) to promote degradation of the kinase (Gao et al. 2008). This dephosphorylation has recently been shown to provide a quality control mechanism to ensure that only functional PKC accumulates in the cell (Baffi et al. 2019). PHLPP also regulates cell cycle progression via activation or inhibition of several kinase targets. PHLPP dephosphorylates the hydrophobic motif of S6K1 (Thr 389) to inhibit protein translation and cell growth (Liu et al. 2011) and Mst1 at an inhibitory site (Thr 387) to promote apoptosis (Qiao et al. 2010). In addition, PHLPP1 dephosphorylates SGT1 at four conserved residues (Ser 17, Ser 249, Ser 289, and Thr 233) to facilitate kinetochore assembly during mitosis (Gangula and Maddika 2017). Finally, PHLPP1 dephosphorylates AMPK at an activating site (Thr 172) in myoblasts to induce ER stress (Behera et al. 2018), a phenomenon frequently accompanied by insulin resistance and diabetes (Hotamisligil 2010). This suggests a potential role for this phosphatase in the impairment of insulin signaling pathways.

The repertoire of PHLPP substrates is expanding to include non-kinase substrates. Nuclear-localized PHLPP1 restrains RTK signaling epigenetically by inhibiting histone phosphorylation and acetylation, thereby dampening transcription of RTKs

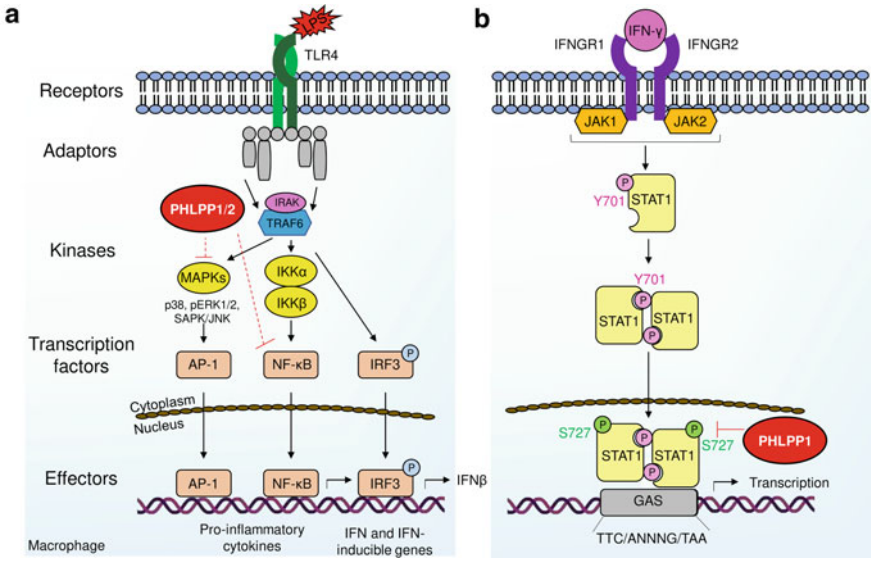

Fig. 3 PHLPP regulation of TLR and IFN-γ signaling. **a** TLR4 engagement activates MAPKs and IKKs through various adaptor proteins; in turn, these kinases promote the nuclear translocation of the transcription factors AP-1 and NF-κB to regulate transcription. TLR4-activated TRAF6 also activates and translocates IRF3 to the nucleus. Both PHLPP1 and PHLPP2 may inhibit TLR4 signaling by dephosphorylating and inactivating p38 (observed in RAW 264.7 cells), Erk1/2 (MAPKs), and NF-κB. **b** IFN-γ acts through its receptor to activate JAK-STAT1 signaling. Phosphorylated STAT1 (Tyr 701) homodimerizes and translocates to the nucleus, where it is further phosphorylated at Ser 727 for full transcriptional activity at promoter elements such as GAS (IFN-γ-activated sequences). In macrophages, PHLPP1 dephosphorylates STAT1 at Ser 727 to oppose its full activation

such as EGFR (Reyes et al. 2014). Thus, PHLPP1 controls two key oncogenic signaling pathways downstream of RTKs, PI3K and Erk1/2, in turn suppressing cell proliferation. Furthermore, the recent finding that PHLPP1 dephosphorylates the transcription factor STAT1 on Ser 727 to suppress its transcriptional activity (Figs. 2 and 3) cements a new role for PHLPP1 in innate immune control (see Sect. 2) (Cohen Katsenelson et al. 2019).

1.3 PHLPP Relevance in Disease

Given its cell cycle-centric regulatory role, it is unsurprising that PHLPP deregulation is associated with multiple pathologies. PHLPP1 mRNA or protein loss is associated with prostate cancer (Chen et al. 2011), chronic lymphocytic leukemia (CLL) (Ouillette et al. 2008), colorectal cancer (Liu et al. 2009; Li et al. 2013), glioblastoma (Warfel et al. 2011), and breast cancer (Qiao et al. 2007), among others. Interestingly,

PHLPP2 expression is elevated during prostate cancer metastasis (Nowak et al. 2015), a paradox resolved by the finding that PHLPP2 can dephosphorylate and stabilize Myc in advanced stages of disease (Fig. 2) (Nowak et al. 2019). Conversely, PHLPP upregulation is also associated with disease: diabetic patients have higher levels of PHLPP1 in skeletal and adipose tissue (Andreozzi et al. 2011; Cozzone et al. 2008). Moreover, PHLPP has other documented functions with potential disease relevance, including circadian regulation (Masubuchi et al. 2010), regulation of chondrocyte proliferation and bone morphogenesis (Hwang et al. 2018; Bradley et al. 2013, 2015), and cardiomyocyte homeostasis (Miyamoto et al. 2010). Finally, PHLPP controls regulatory T cell function (see Sect. 3.1), and its modulation of cell survival in CLL suggests potential involvement in B cell homeostasis as well (see Sect. 3.2).

2 Role of PHLPP in Innate Immunity

Macrophages are key innate immune effector cells. Their expression of PRRs and cytokine receptors enables a critical function in immune homeostasis: the collective detection of danger. Upon exposure either to bacterial products such as lipopolysaccharide (LPS) via the PRR Toll-like receptor 4 (TLR4) or to proinflammatory cytokine signals such as interferon (IFN)-γ via its receptor IFNGR, macrophages activate signaling cascades to aid immune activation and pathogen clearance (Lu et al. 2008). Signal transduction via mitogen-activated protein kinases (MAPKs) and inhibitor of nuclear factor κB (IκB) kinases lead to activation of the transcription factors activator protein 1 (AP)-1, nuclear factor-κB (NF-κB), and interferon regulatory factor (IRF) 3. This ultimately results in the production of prototypical inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12, and type I IFNs (Fig. 3a), among other functions (Lu et al. 2008). To drive the resolution of inflammation, these classically activated macrophages switch to an alternatively activated phenotype characterized by expression of IL-4, IL-13, IL-10, and transforming growth factor (TGF)-β (Nathan and Ding 2010; Spite et al. 2014; Sugimoto et al. 2019). Excessive classical macrophage activity can lead to chronic inflammation and tissue damage, whereas protracted reprogramming of alternatively activated macrophages can favor fibrosis or tumor development (Mantovani et al. 2002; Gieseck et al. 2018). Perhaps the best characterized function of PHLPP1 in macrophages is its regulation of LPS-mediated activation (Cohen Katsenelson et al. 2019; Alamuru et al. 2014), though analogous research in the macrophage response to IFN-γ has begun to shed light on a broader role for PHLPP1 in innate immune control (Alamuru et al. 2014).

2.1 Transcriptional Regulation of PHLPP During Inflammation

Macrophages express both PHLPP1 and PHLPP2, and mounting evidence supports a key role for the PHLPP1 isozyme in these cells. Parsa and colleagues first described that macrophage exposure to LPS decreased PHLPP1 at both the mRNA and protein levels (Alamuru et al. 2014). This may be a more global phenomenon, as this effect has also been observed in non-immune cells such as the Caco-2 intestinal epithelial cell line (Wen et al. 2015). Although the molecular mechanism underlying LPS-driven PHLPP1 downregulation is still not fully understood, a recent study implicated a role for the transcription factor specificity protein 1 (SP1), previously been shown to be depleted during LPS stimulation (Alamuru-Yellapragada et al. 2017; Ye and Liu 2001, 2007, 2002). Analysis of the *PHLPP1* promoter region revealed potential binding sites for various inflammation-related transcription factors, including SP1, STAT1, STAT3, STAT5, and NF-κB (Alamuru-Yellapragada et al. 2017). In support of these in silico findings, SP1 overexpression in the RAW 264.7 macrophage-like cell line results in LPS-dependent recruitment of SP1 to the *PHLPP1* promoter and its transcriptional activation. This effect is synergistically enhanced when SP1 is co-expressed with its coactivators p300 and CREB-binding protein (Tsai et al. 2000; Du et al. 2014), which are also involved in LPS-regulated gene transcription. In contrast, depletion of SP1 reduces *PHLPP1* promoter activity and hence transcription and translation, which is reversed by reintroducing SP1 in LPS-treated macrophages. Taken together, PHLPP1 expression is driven transcriptionally by SP1, which is downregulated by LPS. Of note, this regulatory pathway is conserved in non-immune cells, including several human melanoma cell lines: methylation of the *PHLPP1* promoter in these cells prevents SP1 binding and decreases PHLPP1 expression (Dong et al. 2014).

In addition to macrophages, PHLPP1 is transcriptionally regulated in chondrocytes during inflammation. Histone deacetylase 3 (HDAC3) expressed by mouse chondrocytes binds the *Phlpp1* promoter to reduce its transcription and thus protein expression, ultimately facilitating AKT signaling, matrix production, and chondrocyte proliferation (Bradley et al. 2013). Conversely, human chondrocytes from patients with osteoarthritis, characterized by matrix loss, upregulate *PHLPP1* transcription as a result of epigenetic regulation. Chondrocyte expression of PHLPP1 mRNA and protein is inducible by the inflammatory cytokines IL-6 and TNF-α as well as osteoarthritis-associated reactive oxygen species via demethylation (Bradley et al. 2016).

Overall, PHLPP is transcriptionally regulated by multiple inflammatory stimuli, including bacterial components and cytokines, but the outcomes and mechanisms underpinning these changes are cell- and context-dependent. A more thorough characterization of PHLPP expression in inflammation and homeostasis and the resulting downstream effects in different cell types is needed.

2.2 PHLPP Regulation of TLR4- and IFN-γ-Mediated Signaling

As discussed above, inflammation regulates PHLPP expression to alter cellular outcomes, but emerging evidence suggests that PHLPP phosphatase activity also regulates inflammatory signaling. This regulatory function of PHLPP is particularly evident in signaling cascades downstream of TLR4, which recognizes components of Gram-negative bacteria such as LPS and the inflammatory cytokine IFN-γ.

Normal signal transduction in macrophages downstream of TLR4 and the IFN-γ receptor (IFNGR) involves multiple players. Following TLR4 engagement by LPS, the transcription factor IRF3 induces the expression and secretion of type I IFNs (Gao et al. 1998; Sakaguchi et al. 2003; de Weerd et al. 2007). Once released, IFNs bind to type I IFN receptor complexes to mediate their biological activities in an autocrine and paracrine manner. Additionally, IFN-γ binding to its receptor recruits JAK and STAT1, resulting in the phosphorylation of STAT1 at Tyr 701. This phosphorylation event induces STAT1 homodimerization and nuclear translocation, enabling STAT1 recruitment to IFN-γ-activated sequence (GAS) elements in the promoters of a subset of IFN-γ-responsive genes. Once bound to DNA, STAT1 is further phosphorylated at Ser 727 to enhance its transcriptional activity (Fig. 3b) (Stark et al. 1998; Muller et al. 1994). In addition to inflammatory cytokine secretion, macrophages activated by either LPS or IFN-γ ultimately also produce nitric oxide (NO) in an iNOS (inducible NO synthase)-dependent manner to mediate a wide range of important functions (Bogdan 2001). PHLPP1 has recently been shown to oppose STAT1 function by specifically dephosphorylating Ser 727, unveiling a key role in negatively regulating innate immunity otherwise driven by LPS and IFN-γ (Cohen Katsenelson et al. 2019; Alamuru et al. 2014).

In the RAW 264.7 macrophage-like cell line, Alamuru *et al.* found that PHLPP1 restrained LPS- and IFN-γ-mediated responses (Alamuru et al. 2014). Upon activation by either LPS or IFN-γ, PHLPP1 suppresses iNOS induction by reducing both IFN-β production and STAT1 phosphorylation at Ser 727 (Alamuru et al. 2014). The mechanism by which this occurs varies by stimulus: PHLPP1 opposes LPS-induced iNOS by inhibiting p38 phosphorylation and IFN-γ-activated iNOS by inhibiting Erk1/2 phosphorylation (Alamuru et al. 2014). The finding that PHLPP1 loss results in increased Erk1/2 phosphorylation at its activation loop sites (Cohen Katsenelson et al. 2019; Reyes et al. 2014) supports a negative regulatory role for PHLPP in Erk1/2 signaling.

Studies in primary macrophages confirmed PHLPP1 as a key regulator of STAT1 activity downstream of inflammatory signaling (Cohen Katsenelson et al. 2019). Specifically, upon in vitro activation with the LPS component Kdo2-lipid A (KLA), PHLPP1-deficient macrophages exhibited an increase in STAT1 phosphorylation at Ser 727 but not at Tyr 701. In addition, biochemical analysis revealed that PHLPP1 directly binds to STAT1 and selectively dephosphorylates Ser 727 without affecting the Tyr 701 site. This regulation of STAT1 depends on both PHLPP1's catalytic activity and nuclear localization, the latter driven by the NLS within its

NTE region (Cohen Katsenelson et al. 2019). Transcriptome analysis of PHLPP1-deficient bone marrow-derived macrophages after KLA challenge revealed increased induction of genes involved in the innate immune response. Nearly half of the upregulated genes in these cells contained promoters with a consensus STAT binding motif, and the absence of PHLPP1 enhances STAT1 binding and transactivation of its target gene promoters, such as *Cd69*, *Ifit2*, and *Gbp5* (guanylate-binding protein 5). PHLPP1 also suppresses expression of the STAT1-dependent genes *Socs1*, *Socs3*, *Ccl4*, and *Cxcl10* (Ohmori and Hamilton 2001; Ramana et al. 2001). Furthermore, gene ontology analysis revealed that PHLPP1 also dampens the NF-κB branch of TLR4 signaling. Other gene ontology terms altered by PHLPP1 in TLR4-stimulated bone marrow-derived macrophages encompass many cellular processes, including: cellular receptors, kinases/phosphatases, G-protein-related genes, lipid metabolism, cellular import/export, transcription, protein folding modification, cell adhesion, nucleotide metabolism, extracellular matrix, response to toxins, and immune response. Taken together, PHLPP1 negatively regulates STAT1 transcriptional activity and restrains inflammatory processes in macrophages. It will be interesting to determine whether PHLPP1 is involved in the differentiation of any of the documented alternatively activated macrophage phenotypes and whether PHLPP1 acts similarly in other innate immune cells in response to LPS or IFN-γ.

TLR4 signaling can also be activated by free fatty acids, triggering inflammatory pathways that play a critical role in the development of obesity-associated insulin resistance and type 2 diabetes (Hirosumi et al. 2002; Shi et al. 2006). Genetic studies have identified PHLPP polymorphisms associated with type 2 diabetes (Andreozzi et al. 2011; Turki et al. 2013; Meigs et al. 2007). Obese and diabetic patients have also been found to have increased levels of PHLPP1, but not PHLPP2, in skeletal muscle and adipose tissue (Andreozzi et al. 2011; Cozzone et al. 2008). The finding that PHLPP1 suppresses inflammation upon TLR4 stimulation in macrophages (Alamuru et al. 2014; Cohen Katsenelson et al. 2019) is a new concept, suggesting that these two discoveries may be related. Understanding the molecular and cellular mechanisms of how PHLPP1 regulates insulin signaling through AKT versus inflammatory signaling should be addressed. This research may open new avenues for controlling the low-grade inflammation that constitutes the *sine qua non* of type 2 diabetes, obesity, and other associated metabolic disorders.

2.3 *PHLPP Regulation of the Immune Response upon Bacterial Infection*

Antimicrobial resistance is escalating worldwide as a result of increased antibiotic use and the consequential emergence of competitively advantageous mutations in bacteria (Laxminarayan et al. 2013). Gram-negative pathogens such as *Escherichia*

coli, Salmonella typhimurium, and *Klebsiella pneumoniae* are responsible for potentially fatal infections and have developed various strategies to enable host persistence and immune escape (Laxminarayan et al. 2013; Monack et al. 2004). Uncovering potential therapeutic targets for these bacterial infections and the ensuing host immune response are thus of prime importance. In this context, several reports suggest PHLPP may regulate the antimicrobial immune response.

In macrophages, PHLPP1 elevates lysosomal activity during phagocytosis, suggesting an important role for this phosphatase in bacterial clearance (Fischer et al. 2019) as phagocytosis is a primary mechanism by which macrophages eliminate invading pathogens. Microbes recognized by PRRs are engulfed in phagosomes, which fuse with lysosomes to form a phagolysosome; the acidity and degradative enzymes within the lysosomes destroy the contents of the phagolysosome (Uribe-Querol and Rosales 2017). The circulating adipokine leptin, which activates the mTORC2-AKT signaling axis, has been shown to control phagocytosis via PHLPP1 in macrophages infected by *S. typhimurium.* Genetic and pharmacological inhibition of leptin signaling improves bacterial clearance by augmenting macrophage lysosomal function, and this process is mediated by PHLPP1-dependent AKT dephosphorylation (Fischer et al. 2019). Notably, PHLPP1 can increase lysosomal activity in other contexts, such as in the maintenance of cellular homeostasis: PHLPP1 controls chaperone-mediated autophagy by its association to the lysosomal membrane and enables lysosome-dependent clearance of protein aggregates (Arias et al. 2015). In light of the link between PHLPP1 and mTORC2-AKT-mediated microbial phagocytosis, it will be of particular interest to explore the outcomes of PHLPP1 function in bacterial infection in vivo.

The relevance of PHLPP1 in the immune response in vivo has also been investigated in a mouse model of sepsis. Sepsis is a systemic inflammatory response to bacterial infection characterized by a massive production of cytokines that triggers tissue injury and life-threatening multiorgan failure (Salomao et al. 2012; Glauser et al. 1991; Parrillo 1993; Engelberts et al. 1991). Currently, sepsis is one of the leading causes of death, but the factors contributing to dysregulation of the immune system and poor clinical outcomes remain elusive. A recent study found that PHLPP1-deficient mice were protected from endotoxin-driven (LPS) and bacterial (*E. coli*) sepsis. Specifically, mice lacking PHLPP1 exhibited reduced serum levels of the proinflammatory cytokines IL-6 and IL-1β following LPS injection (Cohen Katsenelson et al. 2019). These findings are in apparent contrast to the aforementioned in vitro work in which PHLPP1 was found to dampen the inflammatory phenotype of macrophages via STAT1 inhibition (Cohen Katsenelson et al. 2019; Alamuru et al. 2014). This discrepancy may be attributed to cell type-specific functions mediated by PHLPP1 in septic shock induced by Gram-negative bacteria. For instance, in another study examining the role of PHLPP in LPS-stimulated neutrophils, neutrophils lacking PHLPP exhibited improved migratory function in vivo and in vitro (Ran et al. 2019). This unique effect of PHLPP1 loss in neutrophils could fine-tune the immune response at a systemic level and contribute to the effect observed in the in vivo model of sepsis.

To date, our mechanistic understanding of the protective role of PHLPP1 deficiency during bacterial or endotoxin-induced septic shock is incomplete. Further experimentation in *Phlpp1*$^{-/-}$ animals, as well as work in animals with myeloid cell-specific PHLPP1 deficiency, will help characterize the effects of this phosphatase. Additionally, given the cell type-specific functions of PHLPP1, a detailed examination of other innate immune cells such as neutrophils, dendritic cells, and natural killer cells may clarify in vivo outcomes. Finally, based on the findings in mice, PHLPP1 may be a promising potential therapeutic target for sepsis by alleviating excessive inflammation and thus mitigating the multiorgan failure associated with septic shock.

3 Role of PHLPP in Adaptive Immunity

The adaptive immune system, comprising B cells and T cells, amplifies innate immune responses in an antigen-specific manner. Following the induction of innate immunity, antigen-presenting cells, such as macrophages, B cells, and dendritic cells, process and present antigens in the form of peptides to T cells. Mounting evidence supports a key role of PHLPP in regulating adaptative immunity.

3.1 PHLPP Signaling in CD4$^+$ T Cells and Regulatory T Cells

T cell activation requires both cognate antigen recognition via their TCR and a costimulatory signal, such as engagement of CD80 or CD86 to CD28 on T cells. The nature of these stimuli as well as the microenvironmental context determine the effector functions of CD4$^+$ and CD8$^+$ T cells following their activation (Lever et al. 2014; Geginat et al. 2014; Arens and Schoenberger 2010). T cell subsets exhibit a certain degree of functional plasticity and primarily contribute to adaptive immunity by lysing infected or cancerous cells, activating and recruiting other immune cells, and establishing antigen-specific memory with potent recall responses.

PI3K-AKT-mTOR signaling in T cells can be activated by TCR engagement, co-stimulation (such as CD80/CD86 ligation to CD28), and IL-2 signaling (Han et al. 2012). PI3K stimulates AKT phosphorylation at Thr 308 via PDK-1, and mTORC2 promotes phosphorylation of AKT at Ser 473; as previously discussed, phosphorylation at both sites is required for full AKT activation. AKT drives proliferation, metabolic reprogramming, and T cell effector function through a variety of targets, including activation of mTORC1-mediated biosynthesis and inhibition of the quiescence-associated Foxo transcription factors (Manning and Toker 2017).

CD4$^+$CD25$^+$ conventional T cells (Tconvs) from *Phlpp1*-deficient mice exhibit exaggerated TCR-induced AKT phosphorylation at Ser 473 (Patterson et al. 2011).

Phlpp1$^{-/-}$ Tconvs are also refractory to suppression by wild-type CD4^{+}Foxp3^{+} regulatory T cells (Tregs; discussed below) (Patterson et al. 2011). As PI3K-AKT-mTOR signaling drives the differentiation of both Tconvs and CD8^{+} T cells (Han et al. 2012; Kim and Suresh 2013), PHLPP deficiency may lead to their overactivation. To date, however, no loss-of-function polymorphisms in *PHLPP1* or *PHLPP2* have been associated with autoimmunity (Chen et al. 2017).

T cell responses are controlled at least in part by a subset of CD4^{+} T cells known as Tregs, which constitutively express the IL-2 receptor alpha chain CD25 and the master transcription factor FOXP3. Tregs use a variety of mechanisms to suppress unwanted immune activity and are required for the establishment and maintenance of immune homeostasis (Vignali et al. 2008; Sakaguchi et al. 2009; Ferreira et al. 2019). As such, their potential as an adoptive cell therapy to induce tolerance in autoimmune or transplant settings is under active preclinical and clinical investigation (Ferreira et al. 2019; Raffin et al. 2020; MacDonald et al. 2019). Of particular interest is uncovering the mechanisms controlling Treg function, adaptability, and stability in inflammation for the development of next-generation Treg therapies. PHLPP has emerged as a potential regulator of Treg identity, but its specific role is not well defined.

A hallmark of both murine and human Tregs is dampened AKT signaling, specifically reduced phosphorylation at Ser 473 upon TCR- or IL-2-mediated activation, as well as reduced phosphorylation of the AKT targets S6 and Foxo1/3a (Bensinger et al. 2004; Crellin et al. 2007). Pharmacological inhibition of PI3K, AKT, or its downstream target mTORC1 in Tconvs promotes de novo expression of Foxp3 and a Treg-like expression profile in vitro (Sauer et al. 2008); Retroviral overexpression of a constitutively active AKT impairs Treg differentiation in vitro and in vivo (Haxhinasto et al. 2008). Furthermore, T cells genetically deficient in mTOR (both mTORC1 and mTORC2) preferentially differentiate into Tregs in vitro (Delgoffe et al. 2009), though some degree of PI3K and mTORC1 activation is required for Treg function in vivo (Patton et al. 2006; Zeng et al. 2013; Soond et al. 2012; Zeng and Chi 2017). Taken together, attenuated activation of PI3K-AKT-mTOR signaling is essential for the development and function of Tregs.

As PHLPP specifically dephosphorylates AKT at Ser 473, its role as a potential negative regulator of AKT signaling in Tregs became of great interest. Indeed, murine and human Tregs have elevated expression of both PHLPP1 and PHLPP2 at the transcript and protein levels compared to Tconvs (Patterson et al. 2011). Consequently, siRNA-mediated or genetic ablation of *Phlpp1* in murine Tregs restores TCR-induced AKT phosphorylation at Ser 473 to levels similar to those of Tconvs. In human Tregs, however, only the combined siRNA knockdown of *PHLPP1* and *PHLPP2* is sufficient to restore phosphorylation of AKT at Ser 473 (Patterson et al. 2011). This may reflect a species-specific compensatory effect by PHLPP2 for PHLPP1 or a gene dose-dependent phenotype as siRNA only reduced *PHLPP1* and *PHLPP2* expression by half in this context (Patterson et al. 2011).

As a negative regulator of AKT activation, PHLPP1 plays an important role in Treg function. Tregs from *Phlpp1*$^{-/-}$ mice have a reduced capacity to inhibit TCR-activated T cell proliferation in a classical in vitro suppression assay and are unable

to protect mice from colitis induced by adoptive transfer of Tconvs (Patterson et al. 2011). The molecular mechanism underlying this functional impairment is an open question. Additionally, whether PHLPP1 deficiency primarily impairs Treg identity during development or Treg function in the periphery is unknown. *Phlpp1*$^{-/-}$ Tregs do not exhibit abnormal proliferation in response to TCR or IL-2 signals (Patterson et al. 2011), suggesting that other aspects of Treg biology are deregulated with PHLPP1 ablation. Many facets of Treg activation, suppressive function, and lineage stability are under metabolic and transcriptional control by AKT targets such as mTOR (Shi and Chi 2019) and Foxo1/3a (Kerdiles et al. 2010; Ouyang et al. 2010, 2012; Luo et al. 2016). These pathways may also be involved in the defective suppressive capacity of PHLPP1-deficient Tregs. Accordingly, human Tregs treated with a small-molecule pan-PHLPP inhibitor (Sierecki et al. 2010) exhibit reduced mitochondrial membrane potential, implying impaired mitochondrial fitness, though this effect is more pronounced with combined inhibition of PHLPP and the lipid phosphatase PTEN (Chen et al. 2017) (discussed below).

Although PHLPP1 and PHLPP2 are important for in vitro Treg differentiation, their role in Treg development in vivo is less clear. Murine Tconvs treated with TGF-β upregulate Foxp3 expression and acquire Treg suppressive properties; however, this in vitro system is less physiologically relevant in human T cells as neither TGF-β- nor activation-induced FOXP3 expression in human Tconvs is sufficient to confer Treg functions or phenotypic features (Allan et al. 2007; Wang et al. 2007; Rossetti et al. 2015). Tconvs from *Phlpp1*$^{-/-}$ mice are less able to differentiate into Tregs in vitro, and this inhibitory effect is potentiated by *Phlpp2* siRNA knockdown, suggesting functional complementation by PHLPP2 for PHLPP1 loss in this setting (Patterson et al. 2011). Similarly, pharmacological inhibition of PHLPP (both PHLPP1 and PHLPP2) in human naive T cells reduces their propensity to upregulate FOXP3 in the presence of TGF-β (Chen et al. 2017). Mechanistically, TGF-β-activated Smad3 binds the *Phlpp1* promoter to induce its expression (Patterson et al. 2011). As TGF-β has a demonstrated role in the development of both thymus-derived and peripherally derived Tregs in vivo (Chen and Konkel 2015; Savage et al. 2020), it is tempting to speculate that PHLPP1 is required for Treg development in vivo. *Phlpp1*$^{-/-}$ mice, however, exhibit normal frequencies of $CD4^+$, $CD8^+$, and $Foxp3^+$ T cell numbers in the thymus and lymph nodes, suggesting unimpaired development for at least thymus-derived Tregs in a global murine PHLPP1 knockout setting (Patterson et al. 2011). Nevertheless, PHLPP2 may functionally compensate in the absence of PHLPP1, and any T cell-extrinsic effects of PHLPP1 ablation cannot be ruled out.

Multiple phosphatases regulating PI3K-AKT activation may function in conjunction in a redundant manner. As alluded above, PHLPP2 may functionally compensate in part for PHLPP1 loss in T cells. Other phosphatases may also cooperate to control PI3K-AKT signaling. The lipid phosphatase PTEN is another major regulator of PI3K-AKT: PTEN dephosphorylates $PI(3,4,5)P_3$ into $PI(4,5)P_2$ to inhibit downstream PI3K signaling (Lee et al. 2018). Like PHLPP, PTEN is also highly expressed by murine and human Tregs (Chen et al. 2017; Bensinger et al. 2004). Curiously, contrary Treg phenotypes are seen in mice and humans with genetic PTEN deficiency.

Mice with a Treg-specific loss of *Pten* develop systemic lymphoproliferative autoimmunity; *Pten*$^{-/-}$ Tregs sequentially lose CD25 and Foxp3, are more glycolytic, and have impaired mitochondrial fitness (Huynh et al. 2015; Shrestha et al. 2015). In contrast, whereas patients with germline heterozygous loss-of-function mutations in *PTEN* (PTEN hamartoma tumor syndrome (PHTS)) also develop autoimmunity, their Tregs do not downregulate CD25 or other Treg-associated markers and maintain normal S6 phosphorylation (mTORC1 target) and mitochondrial membrane potential (Chen et al. 2017). One explanation may be functional compensation for PTEN loss by PHLPP1/2. In support of this notion, PHLPP1 and PTEN are co-recruited to the immunological synapse upon TCR activation of healthy human Tregs (Chen et al. 2017). It remains to be determined whether a similar phenomenon occurs in Tregs from patients with PHTS and whether PHLPP1 potentially compensates by increased expression, increased recruitment to the synapse, elevated phosphatase function, or some combination therein.

Alternatively, *Pten* in Tregs may be partially haplosufficient and not require functional compensation by PHLPP1/2: the aforementioned mouse models lack both copies of *Pten* in Tregs, whereas patients with PHTS maintain one functional copy. Indeed, mice with a less penetrant Treg-specific deletion of *Pten* (deletion in ~ 95% of Foxp3^{+} Tregs) maintain TCR-dependent Treg suppressive activity in vitro (Sharma et al. 2015). Moreover, the stronger autoimmune phenotype in more penetrant strains of Treg-specific *Pten* deletion suggests that, despite unperturbed expression of PHLPP1/2, PTEN has a nonredundant function in maintaining Treg stability (Huynh et al. 2015). These hypotheses are not mutually exclusive. Overall, further investigation is needed to clarify whether PHLPP1/2 indeed functionally compensates for partial or complete loss of PTEN to dampen PI3K-AKT signaling.

Other PI3K-AKT-targeting phosphatases, such as SHIP-1, SHIP-2, and PP2A, may also cooperate with PHLPP1/2 or PTEN in a phosphatase network at the immunological synapse of Tregs. SHIP-1/2 dephosphorylates PI(3,4,5)P_3 into PI(3,4)P_2 (Pauls and Marshall 2017), whereas PP2A dephosphorylates AKT at Thr 308 as well as other targets, including MAPK, NF-κB, and mTOR, in a context- and cell type-dependent manner (Wlodarchak and Xing 2016). Earlier work suggested that the effects of SHIP-1/2 are largely Treg-extrinsic in mice (Tarasenko et al. 2007), but recent studies have implicated a role for SHIP-1 in human Treg function (Cuadrado et al. 2018; Lucca et al. 2019). Additionally, PP2A plays an important role in Treg development and function in mice and humans, particularly in potentiating IL-2 signals (Apostolidis et al. 2016; Sharabi et al. 2019; Ding et al. 2019). Delineating the contexts and relative contributions of PHLPP1/2 and other phosphatases in Treg function as well as in other T cells will be important for rationally targeting these pathways to treat autoimmunity and cancer.

3.2 PHLPP Signaling in B Cells and Chronic Lymphocytic Leukemia

As with T cells, B cells integrate antigenic signals through their BCR and microenvironmental stimuli to orchestrate their development, activation, proliferation, function, and differentiation (Cyster and Allen 2019). In addition to acting as antigen-presenting cells to activate T cells, B cells can differentiate into plasma cells to secrete antibodies, which neutralize pathogens and toxins, facilitate complement activation and pathogen lysis, and promote phagocytosis. Modulation of PI3K-AKT-mTOR signaling in B cells governs nutrient sensing, metabolic reprogramming, and cell fate (Limon and Fruman 2012; Woyach et al. 2012; Jellusova and Rickert 2016). Aberrant AKT activation downstream of BCR engagement contributes to the survival and expansion of malignant B cells, including CLL (Woyach et al. 2012).

A hint that PHLPP1 may be involved in B cell homeostasis and CLL pathogenesis comes from gene profiling studies finding that *PHLPP1* transcript expression is substantially reduced or undetectable in CLL (Basso et al. 2005; Haslinger et al. 2004; Ouillette et al. 2008; O'Neill et al. 2013). At the protein level, blood and tonsillar B cells from healthy individuals express PHLPP1, whereas CLL cells express tenfold less PHLPP1 (Suljagic et al. 2010). In contrast, PHLPP2 protein expression is unchanged between normal B cells, CLL cells, and B lymphoma cell lines. The primary mechanism for PHLPP1 protein downregulation appears to be transcriptional repression as the majority of CLL samples with low PHLPP1 protein expression have a concomitant reduction in *PHLPP1* transcript (Suljagic et al. 2010). Consistent with its phosphatase activity, reintroduction of PHLPP1 in CLL cells by in vitro-transcribed mRNA reduces BCR-induced AKT phosphorylation at Ser 473 as well as Erk1/2 activation, though basal AKT signaling remains undetectable. Other microenvironmental signals, including CD40L, CpG oligodeoxynucleotides, and CXCL12, also increase Erk1/2 activation in the presence of PHLPP1, but AKT phosphorylation at Ser 473 remains limited (Suljagic et al. 2010). Thus, PHLPP1 may differentially regulate signaling pathways downstream of BCR engagement versus other stimuli. Mechanistically, loss of PHLPP1 in CLL promotes cellular survival by upregulating the anti-apoptotic factor MCL-1 (Suljagic et al. 2010), which is normally controlled by AKT (Longo et al. 2008). It will be interesting to determine whether PHLPP1 protein downregulation is a sufficient initiating factor for the survival and propagation of a malignant B cell clone. Finally, given that PHLPP1 loss contributes to CLL pathogenesis, PHLPP1 activity may also plausibly regulate normal B cell homeostasis.

4 Role of PHLPP in Inflammatory Bowel Disease and Colorectal Cancer

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract arising from a combination of genetic and environmental factors, and it is generally classified as Crohn's disease or ulcerative colitis depending on the location of inflammation (Wallace et al. 2014). In a mouse model of dextran sodium sulfate (DSS)-induced colitis, combined deficiency of PHLPP1 and PHLPP2 had a protective effect by increasing AKT activity and consequently reducing epithelial cell apoptosis (Wen et al. 2015). In intestinal epithelial organoids, the absence of PHLPP1/2 upregulates AKT and prevents TNF-α-induced apoptosis (Wen et al. 2015); TNF-α is a major cytokine involved in the development of IBD (Wang and Fu 2005). Although unhindered AKT activity maintains intestinal epithelial cell homeostasis in the context of acute colitis, protracted inflammation and prolonged PI3K-AKT signaling can result in pathology. Indeed, both PHLPP1 and PHLPP2 are downregulated upon inflammatory stimuli via proteasomal degradation, and both isozymes are similarly reduced in colon samples from patients with IBD (Wen et al. 2015). Therefore, although inflammation-driven PHLPP1/2 downregulation initially protects from intestinal epithelial cell apoptosis, its prolonged absence and concomitantly sustained AKT activity may drive the progression of IBD (Wen et al. 2015).

In addition to epithelial cell damage and compromised intestinal barrier integrity, alterations in innate and adaptive immunity have been implicated in the pathogenesis of IBD (Wallace et al. 2014). As mentioned previously, PHLPP-deficient neutrophils exhibit augmented migration in vivo and in vitro and decreased expression of the proinflammatory factors IL-6, IL-1β, and TNF-α. This phenotype is accompanied by enhanced phagosome-lysosome fusion and increased phosphorylation of known PHLPP1 substrates, namely AKT and STAT1, as well as increased Erk1/2 phosphorylation (Ran et al. 2019). The adoptive transfer of neutrophils lacking PHLPP into wild-type mice is sufficient to improve mucosal homeostasis and alleviate symptoms associated with DSS-induced colitis (Ran et al. 2019). Thus, PHLPP is an important player in IBD by modulating not only intraepithelial cell homeostasis but also neutrophil function. Exploring the role that PHLPP plays in other immune cell types in models of colitis may shed light on the molecular mechanisms underlying IBD pathogenesis.

IBD is one of the critical risk factors contributing to colon cancer in humans (Beaugerie and Itzkowitz 2015). Immunohistochemical staining of colorectal tumor samples reveals that both PHLPP1 and PHLPP2 expression are frequently lost, suggesting a tumor-suppressive role for PHLPP isozymes (Liu et al. 2009; Li et al. 2013). This role was further characterized by studies in which knockdown of endogenous PHLPP in cancer cells significantly increased their proliferation rate, and ectopic expression of either PHLPP isozyme interfered with important checkpoints of the cell cycle and reduced cellular growth (Liu et al. 2009). PHLPP-mediated suppression of cellular proliferation is mainly attributed to its negative regulation of AKT signaling. In addition, tumorigenesis induced by subcutaneous injection of colon cancer cells

into nude mice is suppressed with overexpression of PHLPP (Liu et al. 2009). The maintenance of normal PHLPP levels in the epithelial cells of colonic mucosa is thus essential to suppress tumor development. In this context, the deubiquitinase USP46 stabilizes PHLPP expression by reducing its degradation, and downregulation of PHLPP1 expression correlates with decreased levels of USP46 in colorectal cancer (Li et al. 2013).

Whether inflammatory factors produced by immune cells in a PHLPP1-dependent manner could also contribute to tumor development in the colon is still an open question. Given the strong association between chronic inflammation and tumor development, the repression of PHLPP1 expression may amplify proinflammatory signaling in macrophages, exacerbating aggressive tumor growth in colorectal cancer. In other cancer cells, it has been observed that the presence of PHLPP suppresses the production of proinflammatory cytokines such as TNF-α, IL-17, and IL-1β (Teng et al. 2016). IL-1β and IL-17 in particular can promote tumor progression by recruiting macrophages and myeloid-derived suppressor cells and driving angiogenesis (Yang et al. 2014; Iida et al. 2011; Mantovani et al. 2018). These data indicate that the loss of PHLPP isozymes not only promotes proliferation/survival events by increasing AKT phosphorylation (Brognard et al. 2007; Gao et al. 2005) but may also generate an immune microenvironment to favor tumor growth. Exploring the inflammatory state of colonic macrophages and the relevance of PHLPP in immune cell infiltration in the colon during colitis development may shed light on the role of these phosphatases in the pathogenesis of colorectal cancer.

5 Concluding Remarks

PHLPP isozymes are relatively new players in the immune system, yet several roles have already been established for these phosphatases in inflammation and immunoregulation. Thus far, research in this area has focused primarily on the involvement of PHLPP1, with little known about the functional relevance of PHLPP2. In macrophages, PHLPP1 inhibits the adoption of a proinflammatory phenotype by suppressing TLR4 signaling as well as IFN-γ-driven STAT1 activation and increases phagocytosis via AKT dephosphorylation. PHLPP also regulates the migratory and inflammatory capacity of neutrophils in vivo. Interestingly, whole-body deficiency in PHLPP1 protects mice from sepsis, suggesting that PHLPP1 has differential roles dependent on cell type and context. In adaptive immunity, dampened AKT signaling mediated by PHLPP1 is required for immunosuppression by Tregs, though the underlying mechanisms remain unclear. In these cells, PHLPP1 may operate in conjunction with other phosphatases to maintain Treg stability and function, but further investigation is needed to elucidate these effects. PHLPP expression is tightly modulated by inflammation, and aberrant regulation can drive the pathogenesis of diseases such as B cell CLL and IBD. How the effects of PHLPP on TLR4 and STAT1 signaling affect adaptive immunity is an unexplored area of research. Future work in this rapidly

evolving field to dissect the roles of these isozymes may pave the way for novel therapeutics seeking to control a range of immune-mediated diseases, including sepsis, diabetes, autoimmunity, and cancer.

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PI3K and AKT Isoforms in Immune Cells

PI3K Isoforms in Cell Signalling and Innate Immune Cell Responses

Izabela Galvão, Lirlândia P. Sousa, Mauro M. Teixeira, and Vanessa Pinho

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Abstract Phosphoinositide-3-kinases (PI3Ks) are enzymes involved in signalling and modification of the function of all mammalian cells. These enzymes phosphorylate the 3-hydroxyl group of the inositol ring of phosphatidylinositol, resulting in lipid products that act as second messengers responsible for coordinating many cellular functions, including activation, chemotaxis, proliferation and survival. The identification of the functions that are mediated by a specific PI3K isoform is complex and depends on the specific cell type and inflammatory context. In this chapter we will focus on the role of PI3K isoforms in the context of innate immunity, focusing on the mechanisms by which PI3K signalling regulates phagocytosis, the activation of immunoglobulin, chemokine and cytokines receptors, production of ROS and cell migration, and how PI3K signalling plays a central role in host defence against infections and tissue injury.

I. Galvão · M. M. Teixeira
Immunopharmacology Laboratory, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

L. P. Sousa
Signalling in Inflammation Laboratory, Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil

I. Galvão · V. Pinho (✉)
Resolution of Inflammation Laboratory, Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
e-mail: vpinho@icb.ufmg.br

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1 Introduction

Phosphoinositide-3-kinases (PI3Ks) are enzymes that play an important role in modulating innate and adaptive immune cell function through signal transduction and vesicular trafficking. This class of enzymes controls many cellular processes including proliferation, differentiation, survival and migration (Katso et al. 2001).

The importance of the PI3K signalling pathway in different physiological and pathological processes has been described over the years (Fig. 1). Many studies have associated the function of class I PI3Ks with the aetiology and maintenance of inflammatory diseases and innate immune responses (Vanhaesebroeck et al. 2016). For example, PI3K signalling plays an important role in the initial signal transduction events downstream of G-coupled chemoattractant receptors (e.g. receptors for fMLP, C5a, IL-8, LTB4) leading to migration of innate immune cells, including neutrophils (Jones et al. 2003; Pinho et al. 2007), monocytes (Jones et al. 2003) and eosinophils (Lim et al. 2009) to the site of inflammation. PI3K signalling through tyrosine kinase receptors (e.g. Fc receptor, cytokine receptors and integrins) is required for phagocytosis, migration and ROS production in neutrophils (Beemiller et al. 2010; Boyle et al. 2011). Moreover, PI3K signalling mediates homing and distribution of NK cells

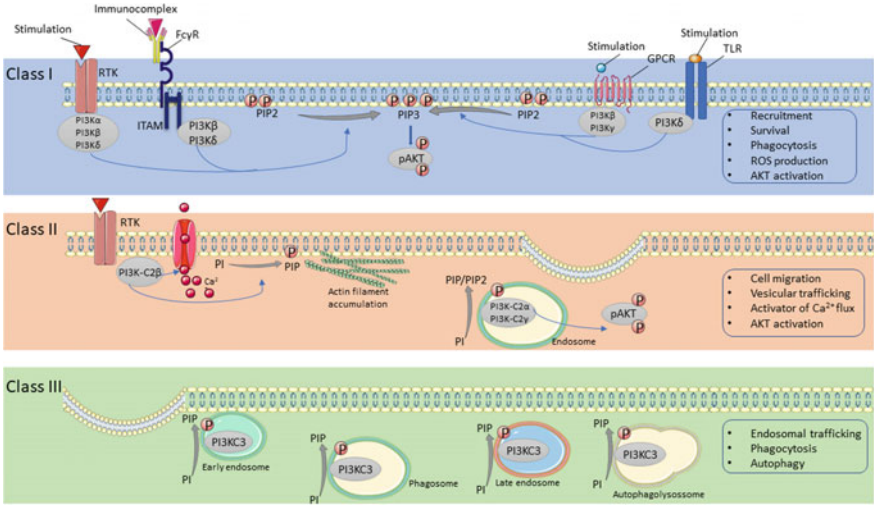

Fig. 1 PI3K isoforms and their function on neutrophils. Class I contains four isoforms: PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ, which produce PI(3,4,5)P3 (PIP3) through activation of receptor tyrosine kinases (RTKs), G-protein-coupled receptors (GPCR), Fcγ receptor and Toll-like receptor (TLR). Class I isoforms promote PIP3 accumulation and AKT pathway activation. Class II contains three isoforms PI3K-C2α, PI3K-C2β and PI3K-C2γ that produce PI(3)P (PIP) and PI(3,4)P2 (PIP2) in distinct cellular compartments. Class II isoforms mediate vesicular trafficking, activate Ca^{2+} flux, direct cell migration via formation of actin-rich lamellipodia and control endosomal AKT activation. Class III contains one isoform, PI3KC3 that regulates autophagy, endosomal trafficking and phagocytosis, which all converge at the lysosome for degradation of the vesicular contents

throughout lymphoid and non-lymphoid organs (Saudemont et al. 2009), and also regulates the production of pro- and anti-inflammatory mediators in macrophages, dendritic cells and neutrophils (Hawkins and Stephens 2015). The PI3K pathway coordinates cell survival via increased AKT activity, which is implicated in inhibiting leukocyte apoptosis (Yasui et al. 2002). Upon inhibition of PI3K/AKT, increased leukocyte apoptosis is associated with resolution of inflammation in pre-clinical models of neutrophilic (Galvao et al. 2018; Lopes et al. 2011; Sousa et al. 2010) and eosinophilic (Sousa et al. 2009) inflammation, suggesting an important role of PI3K/AKT on neutrophil and eosinophil survival. Although some studies have described the function of PI3K in different types of leukocytes, the involvement of PI3K in innate immune cell response has been mostly described in neutrophils (Hawkins et al. 2010). Here, we summarize our understanding of how PI3K signalling governs innate immune responses focusing on its role in neutrophil migration, activation and survival and highlighting how the pharmacological strategies that interfere with the PI3K pathway control the inflammatory response. Whenever data are available, the role of particular isoforms of PI3K in neutrophil function will be examined.

2 The General Role of PI3K in Neutrophil-Mediated Inflammation

A significant number of human diseases are associated with inflammation that can be modulated by PI3K, including rheumatoid arthritis, sepsis, allergy, airway inflammation, obesity-related inflammation, atherosclerosis and cancer-associated inflammation (Costa et al. 2011; Vanhaesebroeck et al. 2016) (Table 1). PI3Ks contribute to the pathogenesis of diseases that have an inflammatory component by regulating the production of inflammatory mediators, inflammatory cell recruitment and the effector function and survival of immune cells (Park et al. 2010; Pendaries et al. 2003). Most of our knowledge about PI3K in innate immune cells comes from studies on neutrophils since they express all PI3K isoforms (Hawkins et al. 2010). Furthermore, neutrophils play an important role in innate immune responses by performing effector functions that contribute to host defence against microbial pathogens and during tissue injury-induced inflammation (Andrews et al. 2007).

Most of the knowledge regarding the role of PI3K in inflammatory diseases has derived from studies evaluating pharmacological inhibition of isoform-specific PI3K and/or from the use of conventional knockout, conditional KO and kinase-dead knock-in mice (Hawkins and Stephens 2015). Data obtained in our laboratories using pre-clinical models of neutrophil-mediated inflammation have shown that the PI3K/AKT pathway is involved in neutrophil recruitment to and survival in several inflammatory sites, including the lungs (Pinho et al. 2007; Russo et al. 2011), pleural cavity (Sousa et al. 2010), articular cavity (Galvao et al. 2018; Lopes et al. 2011; Tavares et al. 2019) and peritoneal cavity (Martin et al, 2010). For example, in a

Table 1 PI3K isoforms in inflammatory disorders

Class	Isoform	Function	Diseases associated
Class I	PI3Kα PI3Kβ PI3Kγ	• Production of cytokines by resident cells • Recruitment of neutrophils • Phagocytosis of opsonized particles or bacteria • Sustain NADPH oxidase activity and production of ROS • AKT activation by recognizing immobilized immune complexes	Arthritis (Camps et al. 2005; Lopes et al. 2011), Sepsis (Martin et al. 2010), Asthma (Campa et al. 2018), Pulmonary fibrosis (Campa et al. 2018; Russo et al. 2011), Pleurisy (Pinho et al. 2005; Sousa et al. 2010), IAV infection (Garcia et al. 2018), Cancer (Vivanco and Sawyers 2002), Gout (Galvao et al. 2018; Tavares et al. 2019), GVHD (Castor et al. 2011), Silicosis (Lima et al. 2019), Multiple sclerosis (Rodrigues et al. 2010)
Class II	PI3K-C2α	• Viral replication • Angiogenesis • Metabolic regulation	Cancer (Gulluni et al. 2017; Mavrommati et al. 2016), Type 2 diabetes (Alliouachene et al. 2016; Braccini et al. 2015), Arthritis (Kim et al. 2020), Allergy (Srivastava et al. 2017)
	PI3K-C2β	• IgE-dependent allergy, • Cell migration, • Metabolic signalling pathways	
	PI3K-C2γ	• Vesicular trafficking	
Class III	PI3KC3	• Early endosome, • Phagosome maturation • Autophagy	Lafora disease (Sanchez-Martin et al. 2020)

model of LPS-induced pleurisy, neutrophil recruitment was shown to be dependent on PI3Kγ and AKT activation, and the levels of AKT phosphorylation paralleled the kinetics of neutrophil infiltration into the pleural cavity after LPS challenge (Sousa et al. 2010). Moreover, panPI3K pharmacological inhibition after LPS challenge led to reduced neutrophil accumulation and survival (Sousa et al. 2010). In a mouse model of graft-versus-host disease (GVDH), the absence of PI3Kγ (PI3Kγ$^{-/-}$ mice) or pharmacological inhibition of PI3K by the use of AS605240 was associated with decreased leukocyte rolling and adhesion in mesenteric venules, suggesting that PI3Kγ plays a role in leukocyte influx and activation in the tissue (Castor et al. 2011).

In a pre-clinical model of pulmonary fibrosis induced by bleomycin, PI3Kγ seems to have an important role in the activation of leukocytes, since, after bleomycin instillation in wild-type mice, phosphorylation of AKT and IκBα were increased in lung extracts. In PI3Kγ knockout mice (PI3Kγ$^{-/-}$), pulmonary injury, collagen deposition and expression of pro-fibrotic and pro-angiogenic genes were reduced along with decreased leukocyte accumulation, production of ROS and nitrogen

species (Russo et al. 2011). In contrast with the observations obtained in models of pulmonary fibrosis, lack of PI3Kγ (PI3Kγ$^{-/-}$ mice) was associated with greater disease severity in influenza A virus (IAV) infection. In this model, the absence of PI3Kγ led to reduced antiviral immune responses resulting in higher viral loads and disease severity, suggesting that PI3Kγ is crucial to regulate both antiviral and inflammatory responses (Garcia et al. 2018). The involvement of class I PI3K isoforms in promoting inflammation has been further supported in an acute model of allergic asthma, where treatment with a pan inhibitor of class I PI3K, LY294002, administered intratracheally exhibited anti-inflammatory effects characterized by decreased AKT phosphorylation in whole lung lysates and reduced leukocyte accumulation in bronchoalveolar lavage fluid (BALF) (Duan et al. 2005). Similar to this, inhalation of the pan-PI3K inhibitor CL27c, that mainly targets PI3Kδ and PI3Kγ (Pirali et al. 2017), reduced the number of leukocytes infiltrating the lungs, which was accompanied by decreased production of inflammatory markers (IL-5 and IL-13) and normalized lung function in a model of OVA-induced acute allergic asthma (Campa et al. 2018). Moreover, this treatment restored steroid sensitivity in a model of glucocorticoid-resistant asthma and attenuated bleomycin-induced fibrosis (Campa et al. 2018). Furthermore, the absence of PI3Kγ (PI3Kγ$^{-/-}$ mice) also reduced lung inflammation caused by silica exposure. PI3Kγ knockout mice showed reduced number of neutrophils in BALF, decreased collagen deposition and reduced lung dysfunction induced by silica (Lima et al. 2019). Taken together, these pre-clinical studies reveal an important role of PI3K in lung inflammation, with some of the effects being directly mediated by PI3K activation in neutrophils.

Absence of PI3Kγ (PI3Kγ$^{-/-}$ mice) or isoform-specific pharmacological inhibition also protects against drug-induced liver damage (Lima et al. 2019). In a model of acetaminophen (APAP)-induced liver injury (DILI), absence or inhibition of PI3Kγ lowered myeloperoxidase (MPO), an indirect measurement of neutrophil accumulation on the tissue and reduced necrotic area in the liver. Moreover, mice were protected from DILI when PI3Kγ was absent in haematopoietic and parenchymal cells, highlighting the importance of PI3K in both myeloid and parenchymal compartments. APAP overdose also caused remote lung injury, characterized by marked leukocyte infiltration in the airways and histopathological alterations. Absence or inhibition of PI3Kγ reduced remote lung injury, suggesting that PI3K plays a critical role in the modulation of immune responses triggered by different stimuli (Lima et al. 2019).

Neutrophil accumulation in the synovial cavity is the main component of joint inflammation (Chen et al. 2018) and PI3K seems to be required for neutrophil recruitment to this site. In a model of albumin-induced arthritis (AIA), AKT phosphorylation was increased in cells collected from the joint cavity of immunized mice challenged with methylated bovine serum albumin (mBSA), concomitant to maximal neutrophil accumulation in the joint cavity. Local treatment with a pan-PI3K inhibitor, LY294002, after arthritis induction in mice reduced the number and survival of neutrophils in the joint cavity (Lopes et al. 2011). Additionally, PI3K is involved in joint inflammation induced by monosodium urate crystals (MSU). Pharmacological blockade of PI3Kγ or PI3Kδ resulted in amelioration of MSU-induced joint inflammation by reducing the number of neutrophils, increasing their apoptosis

and clearance in the synovial cavity. Blockade of one isoform or both is sufficient to induce resolution of inflammation, suggesting that PI3Kγ or PI3Kδ are important for neutrophil recruitment and survival in the inflamed joint (Galvao et al. 2018).

3 The Role of PI3K in Neutrophil Activation and Function

The activation and function of neutrophils are regulated by several interactions between inflammatory mediators and pathogen molecules with receptors presented on the cell membrane (Hawkins et al. 2010). These surface receptors are able to recognize microbial invasion and sense environmental cues and include GPCRs, Fc receptors, adhesion molecules and innate immune receptors (e.g. TLR), which are coupled with PI3K-dependent processes (Fig. 1). Activation of these receptors leads to complex cellular signalling pathways, and activation of several transcription factors that culminate in chemotaxis, phagocytosis, cell survival, cytokine release and generation of reactive oxygen species (Futosi et al. 2013; Stephens et al. 2002).

The family of Fc receptors for IgG (FcγRs) are immunoreceptors containing tyrosine-based activation motifs (ITAM) that trigger cell activation and generate a well-balanced immune response. Neutrophils express various FcγRs that are associated with recognition of Ig-opsonized pathogens. The most important neutrophil FcγR is the low-affinity FcγR (Bruhns 2012). There are different classes of FcγR, and humans express FcγRIIA, which contains ITAM motifs in its cytoplasmic tail, and FcγRIIIB, which has no cytoplasmic segment and is coupled to GPI anchor. In contrast, mice express FcγRIII and FcγRIV, both containing an ITAM-coupled cytoplasmic tail. After immunocomplex activation, FcγR triggers similar signalling pathways starting with tyrosine phosphorylation of the ITAM, which phosphorylates SYK-family kinase and leads to activation of PI3K (Futosi et al. 2013). The generation of PI(3,4,5)P3 recruits phospholipase Cγ (PLCγ) and Bruton's tyrosine kinase (BTK) leading to increased intracellular calcium levels and triggering downstream kinases, such as protein kinase C (PKC). Subsequent activation of ERK, p38 and JNK culminates in cell responses including phagocytosis, cytokine release and oxidative burst (Nimmerjahn and Ravetch 2008). Indeed, immune complexes induce neutrophil activation through the PI3Kβ and PI3Kδ isoforms, with a predominant role of PI3Kβ. PI3Kβ deficient mice are protected from FcγR-dependent inflammation induced by autoantibodies (Kulkarni et al. 2011). In neutrophils, activation of FcγR leads to the release of chemoattractants and cytotoxic molecules (Nimmerjahn and Ravetch 2008). In addition, activation of FcγR also has PI3K-dependent phagocytic function, allowing neutrophils to engulf antibody-coated particles (Booth 2006). Class I and III PI3K are involved in the phagocytic process. Class I is required for engulfment of large particles, and Class III is involved in directing phagosome fusion with endosomes/lysosomes (Vieira et al. 2001). Phagosomal cup formation requires PI(3,4,5)P3 accumulation and further recruitment of proteins that control actin cytoskeleton remodelling, which is essential for the phagocytic process. After the phagosomal vacuole is sealed, PI(3,4,5)P3 levels fade, which might be associated

with the action of phosphatases such as SHIP1 (Gillooly et al. 2001; Marshall et al. 2001). There is evidence that PI(3,4,5)P3 is converted to PI(3)P in neutrophils, which is required for activation of the phagocyte oxidase complex (Ellson et al. 2001).

Nicotinamide adenine dinucleotide phosphate-oxidase/Nox2 oxidase complex (NADPH) is a multicomponent enzyme able to transport electrons to molecular oxygen generating reactive oxygen species, which is essential to kill bacteria (Quinn and Gauss 2004). The NADPH complex is assembled at the plasma membrane in response to inflammatory stimuli capable of activating GPCRs, cytokine receptors such as Tumour Necrosis Factor receptors (TNFRs), and Toll-like Receptors (TLRs). The activation of these receptors primes the cell for a more robust activation of the NADPH oxidase complex (El-Benna et al. 2016). Activation of GPCRs on neutrophils can either prime or activate the NADPH complex directly through class I PI3K-dependent signalling (Nguyen et al. 2017). The production of ROS by neutrophils has a key role in defence against infectious agents (Nguyen et al. 2017). There are different pathways that lead to ROS production, and neutrophils primarily utilize the NADPH oxidase complex (Nguyen et al. 2017). This complex is composed by six subunits: two transmembrane proteins, i.e. $gp91^{phox}$ and $p22^{phox}$, and four soluble proteins: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2 (El-Benna et al. 2016). The assembly of the NADPH oxidase complex facilitates electron transfer from cytosol to oxygen, generating superoxide anions. Following recognition of pathogens and phagocytosis, neutrophils produce antibacterial agents including superoxide anion inside the phagosome (El-Benna et al. 2016). Superoxide anions are the first ROS generated that can convert into different oxygen species, including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid (HOCl), all contributing to bacterial death within the phagosome (El-Benna et al. 2016). Both PI3Kγ and PI3Kβ are required for superoxide production (Hirsch et al. 2000; Houslay et al. 2016). Mature bone marrow neutrophils isolated from $PI3K\gamma^{-/-}$ mice display deficient production of PI(3,4,5)P3, reduced activation of AKT and reduced chemotaxis in response to C5a, IL-8 and fMLP in vitro. NADPH oxidase activation is also impaired in neutrophils isolated from $PI3K\gamma^{-/-}$ mice under serum opsonized zymosan and PMA stimulation. In a model of subcutaneous air pouches, neutrophils from $PI3K\gamma^{-/-}$ mice showed impaired recruitment in response to chemokines (Hirsch et al. 2000). PI3Kβ is also required for ROS formation in response to immune complex and integrin engagement in neutrophils. Mature neutrophils isolated from the bone marrow of PI3Kβ-deficient mice and stimulated with IgG opsonized showed a reduction in the amount of ROS produced (Houslay et al. 2016).

Toll-like Receptors (TLRs) are a family of pattern recognition receptors that recognize conserved molecular structures from a variety of microbes. TLR binding may activate PI3Ks. There are two classes of TLR based on their cellular location: cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) and endosome (TLR3, TLR7, TLR8 and TLR9) TLRs. All TLR have a C-terminal cytosplamic domain, also known as Toll IL-1 Receptor (TIR) domain (Botos et al. 2011) that initiates the signalling cascade through the recruitment of signalling adaptor molecules such as MyD88, TRIF, TRAM, TIRAP and SARM (Kenny and O'Neill 2008). PI3K mediates early

signalling events downstream of TLRs thereby modulating their activation (Pourrajab et al. 2015). For example, stimulation of TLR9 induces the expression of adhesion molecules such as L-selectin, oxidative burst and cell survival via PI3K activation (Hoarau et al. 2007). During TLR9-induced inflammation by CpG treatment, neutrophil recruitment into the pleural cavity was reduced in PI3Kγ knockout mice and after pharmacological inhibition using AS605240 (Lima et al. 2019). Moreover, specific inhibition of PI3Kγ using GSK723 reduced the production of pro-inflammatory cytokines and dampened the levels of phospho-AKT in PBMC from healthy donors after CpG stimulation (Lima et al. 2019). Another example of the involvement of PI3K signalling on TLR-mediated neutrophil functions is TLR2. Upon activation, the p85 subunit of PI3K interacts with the tyrosine kinase domain of TLR2, leading to AKT activation and subsequent NFκB transactivation (Arbibe et al. 2000). Activation of AKT is also directly involved in the modulation of p38 and ERK1/2 activation, contributing to the production of pro-inflammatory cytokines. Pharmacological blockade of PI3K using wortmannin does not inhibit NFκB translocation but decreases NFkB p65 subunit phosphorylation, AKT phosphorylation at Ser 473 and pro-inflammatory cytokine expression, suggesting that PI3K regulates TLR2-induced pro-inflammatory cytokine release by neutrophils through increased AKT activation and p65 subunit phosphorylation enhancing NFκB dependent transcription (Strassheim et al. 2004).

Decades of research have demonstrated that PI3K regulates NFκB activation and, consequently, controls cytokine and chemokine release from neutrophils. PI3K is important for the secretion of S110A8/A9 (Ryckman et al. 2004), platelet activation factor (PAF) (Coffer et al. 1998), TNF (Hatanaka et al. 2004), IL-8 (Dunican et al. 2000) and IL-1β (Galvao et al. 2018) in response to a variety pro-inflammatory stimuli. Class IA PI3Ks mediate cytokine production by human neutrophils, and using pharmacological inhibitors of specific isoforms, p110δ was found to be the PI3K isoform involved in CCL3 (MIP-1α) and CCL4 (MIP-1β) production (Fortin et al. 2011). In addition, activation of the Rac-1/PI3K/AKT axis results in caspase-1 activation and IL-1β release (Kuijk et al. 2008). Accordingly, pharmacological inhibition of PI3K using LY294002 attenuated cleaved caspase-1 production and IL-1β expression in lung tissue in a toluene-diisocyanate (TDI)-induced asthma model, suggesting that PI3K might be a regulator of caspase-1 (Liang et al. 2015). In particular, PI3Kγ has been shown to be required for caspase-1 activation and IL-1β release associated with neutrophil migration and activation in a pre-clinical model of gout (Tavares et al. 2019). Using mice deficient in PI3Kγ or mice with a PI3Kγ mutation causing loss of kinase activity, or a PI3Kγ isoform-specific pharmacological inhibition with AS605240, it was demonstrated that ROS production, activation of caspase-1 and IL-1β release were decreased in the joint after injection of MSU crystals, suggesting that PI3Kγ is a key intracellular molecule required for joint inflammation (Tavares et al. 2019).

GPCR are seven-transmembrane proteins that associate with heterotrimeric G proteins, consisting in α, β and γ subunits. Upon receptor activation, the G protein exchanges GDP for GTP causing a dissociation between Gα and Gβγ subunits that triggers the downstream signalling cascade. GPCR can activate PI3K/AKT directly

through Gβγ subunits or indirectly via GPCR transactivation of tyrosine kinase receptors and integrins. Gβγ subunits directly activate p110β and p110γ and indirectly activate p110δ via activation of tyrosine kinase receptor and small GTPase Ras (Vadas et al. 2013; Vanhaesebroeck et al. 2010). Traditionally, neutrophil chemoattractants belong to four biochemically distinct subfamilies, i.e. chemotactic lipids (e.g. leukotriene B_4 or LTB_4), chemokines (CXCL1 to CXCL3 and CXCL5 to CXCL8 in humans), complement anaphylatoxins (C3a and C5a) and formyl peptides (e.g. N-formyl-Met-Leu-Phe or fMLF). All of them act by activating GPCRs and are involved in stimulating neutrophil activation and migration (Metzemaekers et al. 2020).

4 The Role of PI3K in Neutrophil Migration

Cell migration is a multistep process involving changes in the cytoskeleton, cell-substrate adhesion and the extracellular matrix. It can be divided into four mechanistically separate steps: polarization with lamellipodium extension, formation and stabilization of adhesions, cell body contraction and tail detachment (Lauffenburger and Horwitz 1996). Lamellipodium extension involves actin polymerization and Rac activation. Rac is activated by tyrosine kinase receptor and GPCR in a PI3K-dependent manner. PI3K phosphorylates PI(4,5)P2 into PI(3,4,5)P3, which activates Rac and Cdc42. Both Rac and Cdc42 in turn contribute to PI(3,4,5)P3 accumulation at the leading edge of neutrophils (Wang et al. 2002). This positive feedback loop between PI3K, Rac and Cdc42 is required for the maintenance of the polarized morphology in neutrophils, which is a requirement for migration. Rac and Cdc42 play different roles in actin polarization in the leading edge of neutrophils. Rac is required for PI(3,4,5)P3 accumulation and actin polarization whereas Cdc42 controls localization and stabilization of polarized actin (Srinivasan et al. 2003).

PI3K isoforms play a pivotal role in the orientation and stabilization of the leading edge of migrating neutrophils. Pharmacological inhibition of PI3K using wortmannin and LY294002, in HL-60 cells and neutrophils isolated from healthy donors, stimulated with fMLP or a C5a gradient, resulted in impaired accumulation of F-actin, destabilized the formation of pseudopods, prevented activation of AKT and induced irregular cell migration (Wang et al. 2002). In addition, neutrophil migration is dependent on integrin function and polarization of F-actin, which can be regulated by PI3Kγ. Following fMLP stimulation, neutrophils lacking PI3Kγ showed reduced adhesive properties to a variety of surfaces in vitro. In addition, pharmacological inhibition or absence of PI3Kγ reduced the surface expression of the integrin Mac-1 (a heterodimer of CD11b and CD18), potentially further contributing to reduced neutrophil adhesion. Moreover, in response to the chemoattractant fMLP, neutrophils accumulate F-actin in the leading edge, enabling them to move toward the stimulus. This polarization is reduced in neutrophils either lacking PI3Kγ or pharmacologically treated with wortmannin (Ferguson et al. 2007).

The PI3Kδ isoform also contributes to leukocyte chemotaxis (Reif et al. 2004). Pharmacological blockade and/or genetic depletion of PI3Kδ results in a significant decrease in neutrophil attachment and rolling in the activated endothelium (Puri et al. 2004). A selective PI3Kδ inhibitor, IC87114, inhibited F-actin accumulation in the leading edge of neutrophils suggesting that this isoform is required for neutrophil polarization and directional migration (Sadhu et al. 2003). However, the PI3Kδ inhibitor IC87114 did not affect CXCL1-mediated recruiment of neutrophils in the pleural cavity in vivo. These data were not confirmed by wortmannin treatment, which reduced the CXCL1-mediated recruitment of neutrophils, suggesting that a joint action of diverse PI3K isoforms might contribute to neutrophil migration. The exogenous administration of CXCL1 in PI3Kγ-deficient mice treated with the PI3Kδ inhibitor IC87114, or treatment with inhibitors of both isoforms, IC87114 and AS605240, reduced neutrophil recruitment into the pleural cavity (Pinho et al. 2007). PI3Kδ and PI3Kγ appear to have temporally distinct roles in neutrophil migration. PI3Kγ is required for early responses to various chemokines whereas PI3Kδ plays a more important role at a later time point of cell infiltration (Liu et al. 2007).

5 Role of PI3Ks in the Maintenance of Neutrophil Survival

Numerous studies demonstrate that PI3K and their phosphatidylinositol products govern mammalian cell survival (reviewed by (Datta et al. 1999)). The PI3K/AKT axis has direct and indirect effects on intracellular pathways that govern cell survival. AKT acts directly by phosphorylating anti-apoptotic members of the Blc-2 family (BAD and BAX), and indirectly by changing the expression levels of genes that encode members of the cell death machinery (Datta et al. 1999). Unphosphorylated BAD/BAX can form a complex with pro-survival members of Bcl-2 family on the mitochondrial membrane triggering caspase-mediated apoptosis. AKT phosphorylation of BAD on serine 136 and BAX on serine 184 leads to dissociation of Mcl-1 from BAD or BAX in the cytoplasm, preventing them from binding to members of the Bcl-2 family and allowing them to inhibit apoptosis (Milot and Filep 2011). Furthermore, AKT is able to phosphorylate caspase 9 and to inhibit the initial and terminal execution phases of apoptosis (Vanhaesebroeck and Alessi 2000). AKT can also phosphorylate kinases upstream the stress-activated protein kinase, SAPK, promoting cell survival. Moreover, AKT regulates cell survival through phosphorylation of transcriptional factors including Forkhead Box O (FOXO), NFκB, cyclic AMP response element-binding protein (CREB) and Yes-associates protein (YAP). These transcriptional factors regulate cell survival by controlling the expression of pro-survival target genes (Song et al. 2005). It remains to be determined whether specific PI3K isoforms play differential roles in neutrophil survival by activating AKT.

PI3K activity is critical for haematopoietic progenitor survival, and increased AKT activity is required for neutrophil and monocyte development (Buitenhuis et al. 2008). Neutrophil live or death decision is a key control point in the resolution of

inflammation and is regulated by a complex signalling network involving the Bcl-2 family of proteins, NFκB, caspases and PI3K/AKT (Milot and Filep 2011). Studies have shown opposite evidence about the involvement of PI3K activity in neutrophil survival. Activation of PI3K/AKT pathway through many external stimuli in vitro, such as C5a, fMLP, IL-8 and GM-CSF, delayed neutrophil apoptosis (Klein et al. 2000; Lee et al. 1993). Specifically, PI3Kα activity is required for GM-CSF-induced delayed apoptosis in neutrophils (Klein et al. 2000; Yasui et al. 2002). Furthermore, neutrophils stimulated with GM-CSF presented increased expression of the anti-apoptotic protein Mcl-1 and rapid AKT phosphorylation. Inhibition of PI3K using wortmannin or LY294002 reduced the expression of Mcl-1 and reversed AKT phosphorylation. In addition, increasing doses of these PI3K inhibitors induced a progressive increase in neutrophil apoptosis, suggesting that PI3K regulates GM-CSF-mediated neutrophil survival (Epling-Burnette et al. 2001). PI3K mediates spontaneous neutrophil death through AKT deactivation. Neutrophils isolated from human blood and treated in vitro with wortmannin or AKT inhibitor 1, displayed increased cell death, suggesting that deactivation of AKT signalling is essential to induce neutrophil apoptosis (Zhu et al. 2006).

Not only GM-CSF but also interferon-beta (IFNβ) exhibit anti-apoptotic effects on neutrophils from human peripheral blood by inhibiting caspase 3 and 9 activity and maintaining mitochondrial integrity. This effect is dependent on PI3K since treatment with LY294002 blocked the reduction of neutrophil apoptosis. Moreover, neutrophil survival induced by IFNβ was mediated by PKC-δ and NFκB and not by AKT activation (Wang et al. 2003). Parallel with these findings, neutrophils and mononuclear cells harvested from pleural cavity after LPS stimulation in vivo had increased AKT and Mcl-1 expression and decreased expression of caspase 3. Treatment with LY294002 and AKT inhibitor IV reduced neutrophil accumulation in the pleural cavity, decreased Mcl-1 expression and increased expression of caspase-3, leading to increased neutrophil apoptosis (Sousa et al. 2010). In agreement with these reports, LY294002 treatment during articular inflammation in an antigen-induced arthritis model reduced neutrophil numbers in the joint and also increased the number of neutrophils undergoing apoptosis via reduced NFκB activation (Lopes et al. 2011). Indeed, PI3Kγ mediates leukocyte survival since PI3Kγ-deficient mice present increased number of apoptotic cells in the central nervous system (CNS) after MOG_{35-55} immunization in an experimental model of multiple sclerosis (Rodrigues et al. 2010).

There is a considerable redundancy of PI3K isoform involvement in neutrophil survival (Juss et al. 2012). Selective inhibitors of PI3Kγ and PI3Kδ decreased neutrophil numbers and enhanced apoptosis and efferocytosis, suggesting that either isoform is necessary to maintain neutrophil survival (Galvao et al. 2018).

TLR agonists have been found to delay apoptosis of neutrophils and increase their lifespan through NFκB and PI3K activation. Human neutrophils from whole blood stimulated in vitro with LPS (TLR4 ligand), peptidoglycan (TLR2 ligand), R-848 (TLR7/8 ligand) and CpG-DNA (TLR9 ligand), increased AKT phosphorylation and Mcl-1 levels resulting in delayed neutrophil apoptosis. Inhibition of PI3K with wortmannin reversed these effects suggesting that PI3K is required for

TLR-induced neutrophil survival (Francois et al. 2005). Moreover, Mac-1-mediated adhesion prolongs neutrophil lifespan by activating PI3K/AKT-mediated survival pathway. Mac-1 is a member of β2-integrin family that once activated stimulates AKT activity and induces neutrophil survival through PI3K. The involvement of PI3K was confirmed by pharmacological inhibition using wortmannin, which reversed the anti-apoptotic effect of Mac-1 (Whitlock et al. 2000).

In contrast with the previous studies, activation of AKT can be dissociated from anti-apoptotic effects on neutrophils. Although neutrophil stimulation with fMLP or insulin activates AKT, it does not delay apoptosis, suggesting that the anti-apoptotic role of PI3K triggered by fMLP or insulin stimulation might be mediated by different pathways downstream AKT (Hinton and Welham 1999; Scheel-Toellner et al. 2002). In agreement with these observations, another study demonstrated that G-CSF stimulation activates AKT, but this activation is not enough to extend neutrophil lifespan and delay neutrophil apoptosis, suggesting that not all anti-apoptotic effects of PI3K are mediated by AKT (Souza et al. 2013).

6 The Role of PI3K in Other Innate Immune Cells

Besides neutrophils, PI3K isoforms also play a role in other innate immune cells. Pharmacological inhibition of PI3K with LY294002, reduced the release of pro-inflammatory cytokines after stimulation of LPS by THP-1-derived monocytes, THP-1-derived macrophages and by whole blood from healthy volunteers. Additionally, pharmacological inhibition of PI3K impaired the ability of macrophages to migrate and adhere to extracellular matrix (ECM) and endothelial cells in response to LPS (Xie et al. 2014). PI3Kγ deficient mice displayed reduced macrophage migration toward a chemotactic stimuli and impaired accumulation in a peritonitis model, suggesting that PI3Kγ is crucial for macrophage accumulation during inflammation (Hirsch et al. 2000). The involvement of PI3K in macrophage survival was demonstrated by the use of LY294002 in macrophages isolated from the synovium of patients with rheumatoid arthritis. PI3K inhibition induced apoptosis through suppression of Mcl-1 expression (Liu et al. 2006).

PI3Kγ is also required for mast cell activation during inflammation and allergic responses. Bone marrow-derived mast cells from PI3Kγ deficient mice showed reduced release of histamine-containing granules and intracellular calcium concentration upon antigen-IgE stimulation compared with wild-type (Wymann et al. 2003). In vivo, IgE-induced mast cell infiltration into the dorsal skin and ears was abrogated in mice deficient in PI3Kγ and mice expressing the catalytically inactivate subunit p110γ, whereas PI3Kδ was not required, suggesting that PI3Kγ activity is essential for mast cell infiltration (Collmann et al. 2013).

PI3K and its lipid products play an important role in chemokine-stimulated cell migration. Dendritic cells are critical mediators of both innate and adaptive immune responses and migrate into inflammation in response to chemokines. Their

recruitment to sites of inflammation is required for antigen uptake and their migration to secondary organ lymphoid for antigen presentation is crucial for efficient immune responses (Banchereau et al. 2000). Dendritic cells from PI3Kγ deficient mice showed impaired ability to migrate in vitro towards chemokines and in vivo to draining lymph nodes following local antigen administration (Del Prete et al. 2004).

7 Conclusion

PI3K signalling clearly plays an important role in innate immune responses. Although much progress has been made in the field, further studies are needed to understand the contribution of these enzymes to the functions of innate immune cells. Moreover, it will be important to dissect the role of individual PI3K isoforms and how they interact with each other and other molecules to control the innate immune response in different cell types. Together with in vivo pre-clinical studies, further knowledge of the function of different PI3K isoforms during inflammation may guide the development of novel therapies for chronic inflammatory diseases.

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AKT Isoforms in Macrophage Activation, Polarization, and Survival

Ioanna Lapi, Maria G. Daskalaki, Konstantinos Axarlis, Eleni Paflioti, Philip N. Tsichlis, Eleni Vergadi, and Christos Tsatsanis

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Abstract Macrophages display an array of activation phenotypes depending on the activation signal and the cellular microenvironment. The type and magnitude of the

I. Lapi · M. G. Daskalaki · K. Axarlis · E. Paflioti · C. Tsatsanis (✉)
Laboratory of Clinical Chemistry, School of Medicine, University of Crete, 2208, 71003 Heraklion, Crete, Greece
e-mail: tsatsani@med.uoc.gr

I. Lapi · E. Vergadi
Department of Pediatrics, School of Medicine, University of Crete, 70013 Heraklion, Crete, Greece

I. Lapi · M. G. Daskalaki · K. Axarlis · E. Paflioti · C. Tsatsanis
Institute for Molecular Biology and Biotechnology, FORTH, 70013 Heraklion, Crete, Greece

P. N. Tsichlis
Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH 43210, USA

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response depend on signaling molecules as well as on the epigenetic and metabolic status of the cells at the time of activation. The AKT family of kinases consists of three isoforms encoded by independent genes possessing similar functions and structures. Generation of research tools such as isoform-specific gene deletion mutant mice and cells and isoform-specific antibodies has allowed us to understand the role of each kinase isoform in macrophage activation and homeostasis. This chapter discusses the current evidence on the role of AKT kinases in macrophage activation, polarization, and homeostasis, highlighting the gaps in knowledge and future challenges in the field.

Abbreviations

α-KG	α-Ketoglutarate
ACLY	ATP citrate lyase
AMPK	AMP-activated protein kinase
CLRs	C-type lectin receptors
FAD	Flavin adenine dinucleotide
HAT	Histone acetyltransferases
HK	Hexokinase
IRF	Interferon regulatory factor
JAK	Janus kinase
jmjC	Jumonji-C
LTA	Lipoteichoic acid
MerTK	Mer receptor tyrosine kinase
miRs	Micro RNAs
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian Target of Rapamycin
MyD88	Myeloid differentiation primary response gene 88
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NLRs	Nucleotide-binding leucine-rich repeat-containing receptors
ODN	Oligodeoxynucleotide
oxLDL	Oxidized Low Density Lipoprotein
PFKP1/2	Phosphofructokinase1/2
PH	Pleckstrin homology
RLRs	RIG-I-like receptors
ROS	Reactive oxygen species
SAM	S-adenosylmethionine
SHIP1	SH2 domain-containing inositol-5-phosphatase 1
SOCS	Suppressor of cytokine signaling
TAMs	Tumor-associated macrophages
TBK1	TANK-binding kinase 1
TGF-β	Transforming growth factor-beta

TLRs	Toll-like receptors
TRIF	TIR domain-containing adaptor-inducing interferons
VIP	Vasoactive intestinal peptide

1 Introduction

Macrophages respond to pathogens and other noxious stimuli and initiate innate immune responses. Macrophage activation is under the control of signaling cascades that are initiated by TLR and cytokine signals, resulting in the production of pro- and anti-inflammatory mediators. The type and magnitude of the response to those signals depend on the activation of cascades that positively and negatively regulate transcription of pro-inflammatory cytokines. In addition, activation of transcription factors that control negative feedback mechanisms also modulates the magnitude of macrophage activation. A central signaling cascade that negatively regulates macrophage activation is PI3K/AKT. Activation of AKT results in TLR signaling dampening but also in the activation of cascades that diverge the immune response to an anti-inflammatory state.

AKT signaling is mediated by three distinct kinase isoforms, AKT1, AKT2, and AKT3. These kinases possess common but also distinct functions that are discussed here. AKT kinase isoforms are activated by TLRs and differentially contribute to M1 or M2 polarization. AKT-mediated signals also affect macrophage metabolism, thus indirectly altering TLR responses. AKT has been acknowledged as a central participant in regulating anti-apoptotic cascades in multiple cell types including neutrophils and macrophages (Gardai et al. 2004; Linton et al. 2019). This chapter discusses the known functions of AKT kinases and the particularities of each isoform.

2 AKT Isoforms: Structural and Functional Similarities and Differences

Even though most of the literature refers to AKT as a single protein, AKT is a family of three related proteins, AKT isoforms, termed AKT1, AKT2 and AKT3. They are products of three distinct genes which are located in chromosomes 14q32, 19q13, and 1q44, respectively. The three AKT isoforms have similar structures with high homology (81%) and display significant amino acid sequence differences in the last 130 residues (Heron-Milhavet et al. 2011).

All three proteins contain an amino-terminal pleckstrin homology (PH) domain, a central catalytic kinase domain, and a carboxyl-terminal regulatory domain which includes the hydrophobic motif, a feature of the Protein A, G, and C (AGC) group of kinases. They also contain a linker region that connects the PH domain to the catalytic domain. The most conserved region among AKT isoforms is the central

catalytic domain (~90%) of approximately 250 amino acids (Sale and Sale 2008). The N-terminal 30–40 residues of the regulatory domain are homologous in the AKT, S6, SGK, PKA, and c-GMP kinase families, while the linker region is poorly conserved and has no significant homology to any other human protein (Kumar and Madison 2005).

All AKT isoforms are anchored at the plasma membrane through their PH domain which consists of approximately 100 amino acids and forms a small pocket that binds to high-affinity membrane lipid products such as PIP3 and PIP2 (Song et al. 2005). In their central kinase catalytic domain, there is a conserved threonine residue in the activation T loop, Thr 308 for AKT 1, Thr 309 for AKT2, and Thr 305 for AKT3. AKT kinases are partially activated by phosphorylation of these threonine residues (Song et al. 2005). The regulatory domain contains a phosphorylation site in a hydrophobic pocket with a characteristic motif of the AGC kinase family (F-X-X-F/Y-S/T-Y/F, where X is any amino acid) (Sale and Sale 2008; Pearl and Barford 2002) and includes a consensus ATP-binding motif (Sale and Sale 2008; Zhang et al. 2006). Phosphorylation at the serine residue in the C-terminal regulatory domain of AKT kinases (Ser 473 in AKT1, Ser 474 in AKT2, and Ser 472 in AKT3) also partially activates them. Phosphorylation of both the threonine and the serine residues is required for maximum AKT kinase activity (Song et al. 2005). However, there are human AKT1 variants lacking the second phosphorylation site that still get activated, suggesting that their activation is independent of serine/threonine residue phosphorylation in the hydrophobic motif (Song et al. 2005). In macrophages, phosphorylation of Thr 308 and Ser 473 has been observed, mainly due to the availability of antibodies detecting phosphorylation of specific AKT isoforms on these residues (Ieronymaki et al. 2019).

All three AKT isoforms are widely distributed in tissues, but AKT1 is the most ubiquitously expressed (Heron-Milhavet et al. 2011). AKT2 is expressed at a higher level in insulin-sensitive tissues, i.e., liver, skeletal muscle, pancreas, and adipose tissue, while its expression further increases during adipose tissue and skeletal muscle differentiation (Heron-Milhavet et al. 2011). AKT3 is highly expressed in the brain and is lower in other tissues such as lung, placenta, and kidney (Brodbeck et al. 1999). Moreover, AKT1 and AKT2 have different subcellular localization with AKT2 exhibiting a wider subcellular distribution (Calera et al. 1998). In macrophages, all AKT isoforms are expressed and participate in different functions (Vergadi et al. 2017).

Information on the function of each individual isoform became available through AKT isoform-specific deficient mice and also through the development of isoform-specific antibodies. *Akt1*$^{-/-}$ mice exhibit global growth defects (growth retardation) and reduced life duration, but no alterations in glucose homeostasis, exhibiting a phenotype similar to that of IGF1R$^{-/-}$ mice (Cho et al. 2001a; Chen et al. 2001; Peng et al. 2003). In contrast, mice with full ablation of AKT2 develop hyperglycemia, insulin resistance, and development of diabetes (Cho et al. 2001b). *Akt3*$^{-/-}$ mice exhibit reduction in brain weight arising from decreases in both cell size and number, but maintain normal glucose homeostasis and body weight (Easton et al. 2005). On the contrary, double knockout of AKT1 and AKT3 causes embryonic lethality on days 11–12 with severe developmental defects in the cardiovascular and nervous systems

(Yang et al. 2005). Interestingly, *Akt1*$^{-/-}$ *Akt3*$^{+/-}$ mice exhibit several defects in the thymus, heart, and skin and die soon after birth, while *Akt1*$^{+/-}$ *Akt3*$^{-/-}$ mice survive normally, suggesting that AKT1 rather than AKT3 is crucial for embryonic development and survival, but both are necessary for embryo development (Yang et al. 2005). *Akt1*$^{-/-}$ *Akt2*$^{-/-}$ mice die shortly after birth with significant growth deficiency and exhibiting impaired skin, skeletal muscle, and bone development as well as disrupted adipogenesis (Peng et al. 2003). *Akt2*$^{-/-}$ *Akt3*$^{-/-}$ mice survive and develop normally although they are glucose and insulin intolerant and smaller in size than wild-type mice (Dummler et al. 2006). Remarkably, the presence of a single functional allele of AKT1 in *Akt1*$^{+/-}$*Akt2*$^{-/-}$ *Akt3*$^{-/-}$ mice appears to be sufficient for survival (Dummler et al. 2006). Therefore, AKT1 seems to be the critical pro-survival AKT isoform (Linton et al. 2019; Green et al. 2013). Limited studies are available on the effect of multiple AKT isoform deletion. In T cells, AKT1 and AKT2 are essential for their maturation in the thymus and combined ablation of these isoforms results in inhibition of transition from the double-negative (DN) to the double-positive (DP) stage (Mao et al. 2007; Juntilla et al. 2007). In macrophages, combined ablation of AKT1 and AKT2 results in reduced activation of mTOR and inhibition of Lipopolysaccharide (LPS)-induced Interleukin (IL)-10 production (Toda et al. 2020).

3 The Role of AKT in TLR and Cytokine Signaling

Innate immune cells express germline-encoded pattern recognition receptors (PRRs) that recognize evolutionary conserved pathogen-associated molecular patterns (PAMPs) or host danger signals released in response to various stimuli (damage-associated molecular patterns, DAMPs) (Hajishengallis and Lambris 2011; Takeuchi and Akira 2010). These interactions evoke an immune response through the activation of different intracellular signaling pathways, triggering anti-microbial gene induction as well as cytokine and chemokine production (Kumar and Bhat 2016). Several families of PRRs have been characterized, including TLRs, NLRs, RLRs, and CLRs (Gulati et al. 2018). These PRRs are mainly expressed in macrophages, monocytes, dendritic cells (DCs), and natural killer (NK) cells, but they are also present in lymphocytes and non-professional immune cells such as epithelial cells, endothelial cells, and fibroblasts (Takeuchi and Akira 2010). Serine/threonine AKT kinases are activated downstream of PRRs and are considered central mediators of the signaling pathways initiated by both TLR and cytokine receptors.

3.1 TLR-Induced AKT Activation and Downstream Functions

Among PRRs, TLRs have been the most extensively studied. 13 members of the TLR family have been identified to date in mammals, 10 of them in humans and 13 in mice, with TLR1–TLR9 being conserved in both species (Nie et al. 2018). The interaction of a PAMP with a TLR, present on the cell surface or intracellularly, recruits distinct adaptor molecules that trigger intracellular signaling pathways, ultimately culminating in the activation of inflammatory and anti-microbial responses (O'Neill and Bowie 2007). Signal transduction is mediated via activation of two main pathways: the MyD88-dependent pathway, and the MyD88-independent pathway. All TLRs, except for TLR3, signal through MyD88, activating the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and MAPKs (Nie et al. 2018). Upon activation, NFκB translocates to the nucleus and subsequently activates the expression of pro-inflammatory cytokine genes. Activation of the MAP kinase cascade leads to the induction of the Activator Protein-1 (AP-1) transcription factor complex, which is also involved in cytokine gene expression (Takeuchi and Akira 2010). In the alternative MyD88-independent pathway, TLR3 and TLR4 use TRIF, instead of MyD88, to activate IRF3 and NFκB, inducing the production of type I interferon (IFN) and inflammatory cytokines (Gulati et al. 2018). In addition to TLRs, cytokines, chemokines, and growth factors together act as orchestrators of the early innate immune responses, while setting the ground for the subsequent activation of antigen-specific acquired immunity (Mogensen 2009).

In parallel with the aforementioned pathways, the PI3K/AKT signaling pathway is also activated after TLR/IL-1R stimulation. Early evidence has shown that class IA PI3K recruitment is mediated by interaction of the PI3K p85 regulatory subunit with tyrosine-phosphorylated residues on TLR or IL-1R receptor, or by direct association with the adaptor protein MyD88 in several cell types including macrophages and DCs (Koyasu 2003; Ojaniemi et al. 2003). Upon activation, PI3K phosphorylates and activates its downstream target, AKT. Apart from being activated by PI3K, AKT activation is also induced through direct interaction with MyD88 (Akira and Takeda 2004). In addition, studies have brought to light the involvement of adaptor proteins, such as B cell adaptor for PI3K (BCAP) and MyD88-adapter-like (Mal), that link TLR signaling to PI3K/AKT activation in macrophages (Santos-Sierra et al. 2009; Troutman et al. 2012a). In the context of TLR4 signaling, LPS stimulation results in small GTPase Rab8a recruitment, a Rab GTPase that binds directly to a class IB PI3K, PI3Kγ, ultimately leading to the activation of AKT kinases (Luo et al. 2014).

The majority of reports demonstrate that AKT activation limits pro-inflammatory while promoting anti-inflammatory responses in TLR-stimulated macrophages (Vergadi et al. 2017; López-Peláez et al. 2011). In particular, the PI3K/AKT cascade negatively regulates TLR and NFκB signaling and thus restricts the expression of inflammatory genes (Laird et al. 2009; Luyendyk et al. 2008). For instance, PI3K/AKT pathway inhibited NFκB p65 signaling cascade in response to *Escherichia coli* LPS in monocytes, leading to reduced Tumor Necrosis Factor

alpha (TNFα) production (Guha and Mackman 2002). As previously mentioned, BCAP is essential for TLR-mediated activation of PI3K/AKT pathway, while it negatively regulates pro-inflammatory cytokine production (Troutman et al. 2012a). Moreover, TLR2 stimulation by *Porphyromonas gingivalis* LPS also activated the PI3K/AKT pathway, which negatively regulated the induction of inflammatory mediators such as IL-12 and TNFα, but increased the production of the anti-inflammatory cytokine IL-10 (Martin et al. 2003). Additionally, TLR2 activation by *Staphylococcus aureus* (*S. aureus*) resulted in PI3K/AKT pathway induction, which in turn phosphorylated and terminated the pro-inflammatory function of Forkhead box transcription factor O1 (FoxO1) (Wang et al. 2016). In another study, stimulation of the TLR4-PI3K-AKT pathway inactivated FoxO1, with the latter acting as a negative regulator of TLR4-mediated inflammatory signaling (Fan et al. 2010). Upon TLR signaling, PI3K/AKT pathway also inactivates glycogen synthase kinase (GSK)-3β, a serine/threonine kinase that is responsible for pro-inflammatory cytokine production (Guha and Mackman 2002; Martin et al. 2005). Activation of MerTK negatively regulates TLR2 signaling and concomitantly TNFα and IL-6 production, which is induced by LTA, via the PI3K/AKT pathway and SOCS3 protein (Zhang et al. 2016).

In contrast to these studies, the PI3K/AKT pathway has been also shown to act positively in inflammatory gene induction (Lee et al. 2003). An early study indicated that AKT was activated by heat-killed *S. aureus*-mediated TLR2 stimulation through the Rac1-PI3K pathway, resulting in NFκB transactivation (Arbibe et al. 2000). LPS interacts with TLR4, triggering the production of inflammatory mediators via induction of the AKT/NFκB axis (Ojaniemi et al. 2003; Su et al. 2017). In addition, a recent study showed that the TLR-driven PI3K/AKT/mTOR-dependent inflammatory response of U937 macrophage-like cells is inhibited by A549 epithelial cells due to a reduction in the production of soluble cytokines and mediators upon infection with *Mycobacterium tuberculosis* (Yang et al. 2018).

AKT signaling is not only critical for controlling the activity of the NFκB transcription factor, but also for regulating the activation of IRF transcription factors. Upon TLR3 and TLR4 stimulation, AKT, which is the downstream effector of the TRIF/ TBK1 pathway, activates IRF3 (Joung et al. 2011). Furthermore, TLR-mediated activation of PI3K/AKT pathway leads to mTOR activation, which in turn associates directly with MyD88, culminating in the activation of IRF5 and IRF7, master regulators of pro-inflammatory cytokines and IFNα, respectively (Schmitz et al. 2008). Moreover, AKT1 was reported to negatively regulate cytokines produced through both the MyD88- and TRIF-dependent pathways in TLR signaling by suppressing NFκB activation and IRF3 activity independently of its kinase activity (Zenke et al. 2018).

In addition to its implication in pro- and anti-inflammatory cytokine production, AKT signaling downstream of TLRs is triggered by different ligands and regulates distinct functions in macrophages. For instance, TLRs can be recognized and activated by different pathogens, leading to the activation of the AKT pathway, and subsequent increase in macrophage phagocytic capacity (Shen et al. 2010). Additionally, saturated fatty acids, such as lauric acid, or natural bioproducts, like alginate, can also induce TLR activation, leading to NFκB activation, which is PI3K/AKT-dependent

(Lee et al. 2003; Bi et al. 2017). TLR-9 stimulation by CpG ODN induces the phosphorylation of AKT in murine macrophages, resulting in matrix metalloproteinase-9 (MMP-9) and TNFα expression, with potential impact on inflammatory disease development (Lim et al. 2006). AKT phosphorylation by TLRs has been also associated with macrophage function in pathology (Gu et al. 2020a; Singh et al. 2020). An overview of the contribution of AKT in TLR signaling is presented in Fig. 1.

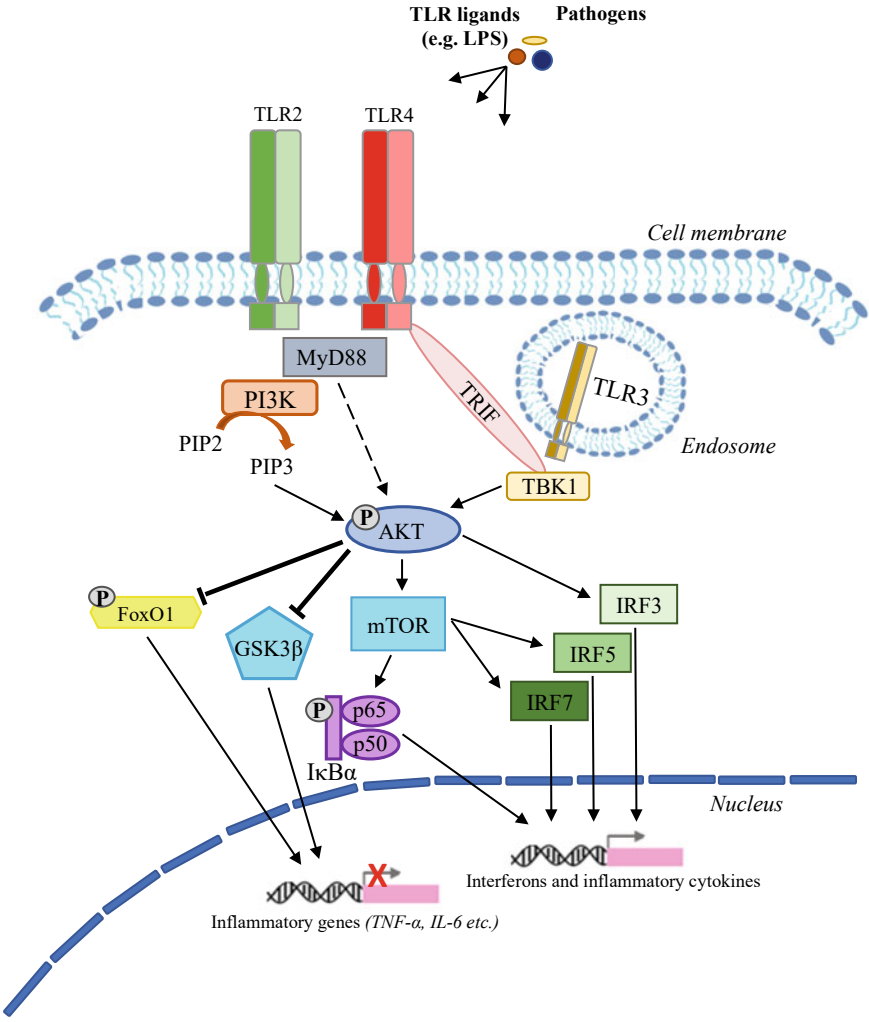

Fig. 1 In macrophages, engagement of TLRs by PAMPs results in PI3K/AKT signaling cascade induction, which is MyD88-dependent or -independent. Subsequently, AKT kinase induces the transcription of interferons and inflammatory cytokines by activating transcription factors such as IRFs and NFκB. On the other hand, several studies indicate that AKT activation suppresses the expression of inflammatory genes by inhibiting the function of FoxO1 and GSK-3β

3.2 Cytokine-Induced AKT Signaling

Cytokines may have autocrine, paracrine, or endocrine functions and they orchestrate the nature of the immune response by regulating distinct cellular functions such as cell growth, differentiation, survival or apoptosis, and metabolism (Zhang and An 2007). The interaction of a cytokine with its cognate receptor rapidly induces tyrosine phosphorylation of the receptor, resulting in Janus kinases (JAKs) recruitment. Activated JAKs propagate the initial signal through the induction of signaling molecules such as STATs, Src kinases, protein phosphatases, and other adaptor signaling proteins, such as Shc, Grb2, and PI3K (Jatiani et al. 2010). Therefore, AKT signaling is activated by different cytokines and regulates transduction of their signals.

In addition to TLR-mediated activation, class IA PI3Ks and AKT are also induced by cytokine receptors which mediate their signals through tyrosine kinases and are resident on the surface of immune cells (Koyasu 2003). Cytokine receptor signaling acts in concert to TLR signals, modulating immune cell functions such as phagocytosis and autophagy. The anti-inflammatory cytokine IL-10 activates the PI3K/AKT pathway, which results in inhibition of autophagy in murine macrophages during starvation (Park et al. 2011). Moreover, exposure of macrophages to IL-4 in conjunction with IgG-opsonized zymosan increases PI3K/AKT activity, contributing to the change in the phagosomal membrane potential (Keijzer et al. 2011). IL-18, a cytokine produced by macrophages in the early stages of the inflammatory response, induces MCP-1 production through the PI3K/AKT pathway in macrophages (Yoo et al. 2005). Another study demonstrated that IL-27 activated the JAK/PI3K/AKT/mTOR cascade, inhibiting IFNγ-induced autophagy, and thereby promoting intracellular survival of *Mycobacterium tuberculosis* H37Rv in macrophages (Sharma et al. 2014). AKT signaling is activated not only by interleukins but also by other cytokine families. Culture of bone marrow-derived macrophages with macrophage colony-stimulating factor (M-CSF) was shown to induce the phosphorylation of AKT on Ser 473 that was barely affected by IL-4 or IL-13 in a cell culture model of human monocytes and primary mouse macrophages (Heller et al. 2008). Stimulation with RANKL promoted macrophage differentiation into osteoclasts that was AKT1/AKT2-dependent (Sugatani and Hruska 2005). The AKT signaling pathway is also activated upon transforming growth factor β (TGF-β) stimulation (Olieslagers et al. 2011; Liu et al. 2019). Moreover, IFN family has also been reported to mediate AKT phosphorylation. IFNγ and IFNβ stimulation of primary human peripheral blood monocytes and THP-1 cells led to the phosphorylation of AKT, regulating monocyte adhesion (Navarro et al. 2003). IFNγ modified TLR2-induced signaling via PI3K-AKT pathway inhibition, which in turn increased GSK3 activity, leading to diminished IL-10 production (Hu et al. 2006). AKT also contributes to signal transduction initiated by several chemokines affecting macrophage migration as well as macrophage metabolism (Chien et al. 2018).

3.3 AKT Isoform-Specific Signaling Downstream of TLRs

Most studies have addressed the role of AKT signaling in immunity as a single unity. However, recent findings have reported that the different AKT isoforms have non-redundant functions in TLR and cytokine signaling. AKT1 may regulate TLR signaling indirectly by controlling IL-1R-associated kinase (IRAK)-M expression, which has been shown to negatively regulate TLR signaling (Kobayashi et al. 2002; Androulidaki et al. 2009). Despite the fact that both AKT1 and AKT2 isoforms are highly expressed in macrophages, *Akt1*$^{-/-}$ macrophages are hyperresponsive to LPS, failing to develop endotoxin tolerance, whereas *Akt2* ablation is associated with macrophage hypo-responsiveness in response to the same stimulus (Arranz et al. 2012). Therefore, the absence of AKT1 contributes to a pro-inflammatory phenotype. AKT1 is also involved in the regulation of microRNAs, which are induced by LPS/TLR4. In particular, AKT1 positively regulates the miRNAs let-7e and miR-181c, while negatively regulates miR-155 and miR-125b in primary mouse macrophages (Androulidaki et al. 2009). As miR-155 positively affects TLR signaling, AKT1-mediated inhibition of miR-155 expression via induction of C/EBPβ leads to a reduction of LPS-induced responses (Doxaki et al. 2015). In addition to miR-155, AKT1 promotes the induction of let-7e, which in turn restricts TLR4 expression (Androulidaki et al. 2009). After LPS stimulation, the induction of miR-29a increased the expression of IL-1β and IL-6 by negatively regulating AKT1 (Tang et al. 2017). Another study showed that VIP rendered macrophages hyporesponsive to LPS by downregulating TLR4 expression via AKT1 (Arranz et al. 2008). On the contrary, AKT2 deficiency resulted in a significant upregulation of the anti inflammatory miRNA miR-146a which suppresses TLR4 signaling and promotes an M2 anti-inflammatory phenotype, protecting mice from acid-induced lung injury (Vergadi et al. 2014). AKT3 is also expressed in macrophages but limited information on its role in this cell type is available. Deletion of *Akt3* in macrophages enhances pinocytosis and low-density lipoprotein uptake (Ding et al. 2017), while full AKT3 deletion in an in vivo model resulted in impaired wound healing and macrophage function in the healing process (Gu et al. 2020b). In the mouse model of atherosclerosis induced by Apolipoprotein E deficiency, lack of AKT3 resulted in increased lipid uptake from macrophages and foam cell formation (Ding et al. 2012).

4 Contribution of AKT Kinases to Macrophage Polarization

Macrophages are able to acquire distinct phenotypes or polarization states due to their inherent plasticity. A polarization state refers to a macrophage activation and phenotypic state at a given point in space and time. This concept of macrophage polarization is an attempt to cluster the different immune responses of macrophages according to the activation stimuli in addition to surface markers macrophages express, production

of specific factors, and biological activities. The stimuli could be either cell-intrinsic or extrinsic and include cytokines, growth factors, fatty acids, prostaglandins, and pathogen-derived molecules (Atri et al. 2018). All these diverse activation triggers are grouped into two major polarization programs, classically activated macrophages or M1, and alternatively activated macrophages or M2. M1 macrophages are activated by TLR ligands or cytokines secreted by Th1 lymphocytes, such as IFN-γ and TNF-α. These macrophages secrete high levels of pro-inflammatory cytokines, such as TNF-α, IL1-β, IL-6, IL-12, and IL-23, promoting Th1 polarization of $CD4^+$ T lymphocytes. In addition, M1 macrophages eliminate ingested microbes through activation of the NADPH oxidase system, followed by generation of ROS (Shapouri-Moghaddam et al. 2018). The detrimental effects of a prolonged inflammatory response are inhibited by regulatory mechanisms driven by the anti-inflammatory function of M2 macrophages. It is widely acknowledged that M2 macrophages include a wide spectrum of different phenotypes depending on the activation signal. Several attempts have been made to classify different types of M2 macrophages. In one of those, based on in vitro experiments, the M2 spectrum consists of four distinct subtypes, M2a, M2b, M2c, and M2d, dependent on the activation signal (Martinez et al. 2008). These M2-like subtypes, induced by Th2 polarizing cytokines, constitute anti-inflammatory macrophages. M2a macrophages, activated by IL-4 and IL-13, promote fibrosis and wound healing and are also termed M(IL-4) in a distinct classification (Murray et al. 2014). M2b macrophages (also termed M(Ic)) are stimulated by exposure to both immune complexes (ICs) and TLR ligands or IL-1 receptor agonists. This is the only M2 subtype that secrets pro-inflammatory cytokines, IL-1β, IL-6, and TNFα but low levels of IL-12. M2b macrophages also produce high levels of the anti-inflammatory cytokine IL-10, and thereby, perform immune-regulatory functions (Murray et al. 2014). M2c macrophages (also called M(IL-10) are stimulated by IL-10, glucocorticoids, and TGF-β, and regulate efferocytosis and tissue remodeling (Murray et al. 2014). Finally, M2d macrophages also referred to as TAMs, are induced by exposure to both TLR and adenosine A2A receptor ligands, or by IL-6, and contribute to angiogenesis and tumor growth (Vergadi et al. 2017; Abdelaziz et al. 2020).

Numerous studies have shown the importance of the AKT signaling pathway in the immune function of macrophages. M1 and M2 polarization states are tightly regulated by the PI3K/AKT pathway and its downstream targets. As previously stated, most reports demonstrate that activation of the PI3K/AKT pathway negatively regulates TLR signaling, inhibiting the production of pro-inflammatory cytokines, and thus, classical macrophage activation (Troutman et al. 2012a; López-Peláez et al. 2011; Luyendyk et al. 2008). However, it is a controversial topic as the PI3K/AKT pathway has also been involved in the production of inflammatory mediators via induction of NFκB, a master regulator of M1 activation (Ojaniemi et al. 2003; Lee et al. 2003; Arbibe et al. 2000; Su et al. 2017).

AKT-dependent mTOR signaling seems to control macrophage polarization. mTOR is comprised of two functionally distinct complexes, mTORC1, and mTORC2. AKT directly activates mTORC1, but also indirectly through inactivation of TSC2, which inhibits the activity of the Rheb GTPase, an activator of mTORC1. mTORC2 phosphorylates and activates AKT, while activated mTORC1 mediates

feedback inhibition to restrict mTORC2 and AKT activity (Memmott and Dennis 2009). mTOR activation via the PI3K/AKT pathway has been shown to promote M2 macrophage polarization (Covarrubias et al. 2015; Rocher and Singla 2013). In accordance with the previous studies, the use of rapamycin that inhibits mTORC1 activity promoted M1 activation (Weichhart et al. 2008). In contrast, peritoneal macrophages from high-fat diet-fed mice, which were Raptor deficient and thus mTORC1 deficient, exhibited decreased inflammatory gene expression through suppression of the inactivation of AKT and subsequent inhibition of Inositol-requiring transmembrane kinase-endoribonuclease 1α (IRE1α)/ c-Jun N-terminal kinase (JNK)/NFκB pathways (Jiang et al. 2014). Additionally, constitutive mTORC1 activity due to Tsc1 deletion skewed macrophages to an M1 phenotype by suppressing AKT activation (Byles et al. 2013). TSC1-deficient macrophages also displayed a hyperinflammatory response to LPS, while the activity of AKT was again decreased (Pan et al. 2012). In line with these reports, another study using TSC1-deficient macrophages demonstrated that AKT-TSC1/2 complex-Ras GTPase axis constitutes a positive feedback loop that promotes inflammatory response during LPS-induced M1 polarization (Zhu et al. 2014). Overall, the observed reduced M2 activation in TSC1-deficient macrophages could be due to attenuated AKT signaling. This polarization defect may also be attributed to the reduced expression of the transcription factor C/EBPβ, which is regulated by AKT (Arranz et al. 2012; Covarrubias et al. 2015). Furthermore, mTORC2 is shown to be essential for maximal activation of AKT after TLR stimulation. Primary macrophages isolated from mice lacking Rictor, a subunit of the mTORC2 complex that phosphorylates AKT, expressed high levels of M1 genes and lower levels of M2 markers in response to TLR agonists (Festuccia et al. 2014). However, macrophages deficient in Rictor were able to be polarized normally into the M2 phenotype after IL-4 stimulation (Byles et al. 2013). Recent evidence suggests that AKT-mTORC1 activation drives macrophages toward an M1 pro-inflammatory phenotype, characterized by high anaerobic glycolysis, fatty acid synthesis, and a truncated citric acid cycle, and not oxidative phosphorylation, which is typical of M2 macrophages (Linton et al. 2019; Geeraerts et al. 2017).

In addition to mTOR, macrophage polarization is also affected by different regulators of the PI3K/AKT pathway. For example, the kinase phosphoinositide-dependent protein kinase 1 (PDPK1) is crucial for AKT activation and its deletion in macrophages increased M1 polarization in adipose tissue (Kawano et al. 2012). Macrophages from myeloid-specific *Pdpk1*$^{-/-}$ mice showed reduced TLR4- and TLR2-induced AKT activity, while exhibiting exaggerated pro-inflammatory gene expression (Chaurasia et al. 2010). Additionally, AMP-activated protein kinase (AMPK) positively regulated AKT activity, promoting an M2-like anti-inflammatory phenotype (Sag et al. 2008). In agreement with the previous study, AMPK was shown to negatively regulate LPS-stimulated inflammatory responses in macrophages through induction of the PI3K/AKT/GSK3β/CREB pathway and inhibition of NFκB activity (Zhu et al. 2015). Moreover, loss of PTEN, which is a major negative regulator of the PI3K/AKT pathway, results in increased AKT activity and the development of an M2 polarization state (Luyendyk et al. 2008; Kral et al. 2016). Loss of SHIP, an inositol phosphatase that hydrolyzes PI3K products thus inhibiting AKT activity,

shifts macrophages toward an M2-like phenotype (Li et al. 2016; Qin et al. 2020; Rauh et al. 2005). However, SHIP-deficient macrophages exhibit increased AKT activation, followed by enhanced inflammatory cytokine response upon infection with *Francisella tularensis* (Parsa et al. 2006).

AKT regulates macrophage activation also via downstream effectors. For instance, GSK3 activity is inhibited by the PI3K/AKT pathway upon TLR stimulation, promoting an M2-like phenotype with reduced secretion of pro-inflammatory mediators and increased IL-10 production (Martin et al. 2005). Furthermore, TLR-mediated AKT activation suppresses the activity of the transcription factor FoxO1, and thereby negatively controls the inflammatory response (Wang et al. 2016; Fan et al. 2010). Several studies have demonstrated that AKT restricts the activity of the M1 regulator, NFκB (Luyendyk et al. 2008; Guha and Mackman 2002), while others have reported that it can be also positively regulated by AKT (Ojaniemi et al. 2003; Arbibe et al. 2000).

Therefore, most of the studies demonstrate that AKT signaling pathway plays a predominant role in macrophage polarization, promoting M2 activation by regulating the inflammatory response. AKT inhibition abrogates the upregulation of M2-associated genes while promoting the induction of M1 genes, leading to elevation in pro-inflammatory cytokine expression (Troutman et al. 2012a, 2012b; Covarrubias et al. 2015; Byles et al. 2013; Rückerl et al. 2012). Nevertheless, studies using knockout gene-targeting approaches of individual AKT isoforms have revealed that each isoform contributes differently to macrophage activation state (Vergadi et al. 2017). For example, let-7e contributes to the LPS hyperresponsiveness observed in *Akt1*$^{-/-}$ macrophages by regulating TLR4 expression (Androulidaki et al. 2009; Curtale et al. 2018). MiR-155 controls the production of inflammatory cytokines by inhibiting the expression of the suppressor of cytokine signaling 1 (SOCS1) (Androulidaki et al. 2009) and the transcription factor C/EBPβ that is master regulator of M2 polarization (Arranz et al. 2012), thereby promoting M1 activation (Bala et al. 2011; Cai et al. 2012; Tili et al. 2007). In addition to these miRNAs, miR-125b and miR-181c are also under the control of AKT1 in LPS-activated macrophages (Androulidaki et al. 2009). According to the work of Arranz and colleagues, AKT1 and AKT2 kinases differentially affect macrophage polarization. Consistent with previous results, genetic ablation of AKT1 resulted in hyperresponsive macrophages to LPS that failed to develop endotoxin tolerance. These LPS-stimulated *Akt1*$^{-/-}$ macrophages were characterized by increased production of NO, TNF-α, and IL-6. In contrast, *Akt2*$^{-/-}$ macrophages were hyporesponsive to LPS stimulation as they acquired an M2-like phenotype based on the increased expression of typical markers of alternative activation, such as *Arg1*, *Ym1*, and *Fizz1* (Arranz et al. 2012). In agreement with this M2-like phenotype, Akt2-deficient macrophages expressed reduced miR-155 and elevated C/EBPβ levels as well as increased LPS-induced IL-10 (Arranz et al. 2012; Nandan et al. 2012). Another study also showed that Akt2 deficiency in macrophages results in an M2 anti-inflammatory phenotype via miR-146a upregulation (Vergadi et al. 2014). Moreover, loss of AKT1 induced bactericidal capacity in macrophages (Arranz et al. 2012; Xu et al. 2013a; Kuijl et al. 2007). In addition, AKT1 has also been reported to mediate repression of TLR4-induced macrophage

activation by adipokines and neuropeptides (Vergadi et al. 2017; Arranz et al. 2008; Zacharioudaki et al. 2009).

5 Role of AKT Kinases in Metabolic and Epigenetic Regulation of Macrophage Activation

A cascade of transcriptional and post-transcriptional signals tightly regulate inflammatory gene expression to ensure fine-tuned macrophage activation and polarization responses (Li et al. 2018). Great interest has risen in exploring potential epigenetic regulators contributing to macrophage activation. Intense research in the field has uncovered a variety of chromatin remodeling enzymes that shape macrophage responses (Daskalaki et al. 2018). Efficient macrophage activation is a process requiring energy and involves metabolic reprogramming. TLR receptor signaling and nutrient-dependent signals mediate these changes through nuclear receptors (PPARγ) and kinases (PI3K/AKT/mTOR) (Artyomov et al. 2016). Phagocytosis, ROS, and pro-inflammatory cytokine production are highly dependent on glycolysis (Mehta et al. 2017), whereas anti-inflammatory (M2 like) responses are rather dependent on oxidative phosphorylation (Kelly and O'Neill 2015).

The metabolic signature in macrophages is tightly associated with epigenetic changes. Chromatin dynamics are under the control of covalent modifications such as methylation, acetylation, ubiquitination, phosphorylation, sumoylation, and ADP ribosylation. The best-studied modifications are the insertion and deletion of methyl and acetyl marks on residues of histone tails regulating chromatin accessibility to the transcription machinery (DesJarlais and Tummino 2016). Chromatin regulation at the epigenetic level is closely linked to immunometabolism, as the establishment of epigenetic modifications is regulated by both immune signaling and metabolic pathways. Metabolites such as SAM and acetyl-CoA act as donor molecules for the induction of methyl and histone marks, respectively, whereas NAD^+, FAD, α-KG and others act as co-factors for reversal reactions to remove histone modifications (Daskalaki et al. 2018), events that occur in all mammalian cell types (Fig. 2).

The AKT/mTOR signaling cascade directly regulates glycolysis by activating the expression of glycolytic enzymes such as HK and PFKP1/2, and by fueling the tricarboxylic acid cycle (TCA) with pyruvate, generated by glucose catabolism (Kim 2018). In recent years, a number of TCA intermediate molecules including acetyl-CoA, succinate, fumarate, and α-KG were found to affect the epigenome by modulating the expression of enzymes that control histone methylation and acetylation (Verberk et al. 2019). Consequently, AKT indirectly regulates macrophage activation at the epigenetic level through downstream targets by controlling the availability of key metabolites (Baardman et al. 2015). HATs catalyze the transfer of an acetyl group from the acetyl-CoA to ε-amino group of a histone lysine residue, therefore acetyl-CoA availability critically influences histone acetylation (Hirsch et al. 2017).

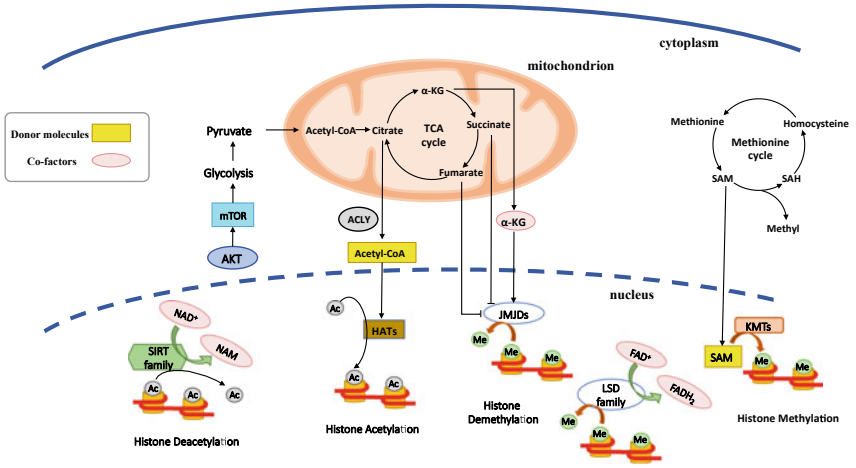

Fig. 2 Macrophage activation and cell metabolism are tightly linked, fine-tuning the initiation and resolution of the immune response. AKT/mTOR pathway directly regulates metabolism by inducing glycolysis, fueling the TCA cycle through pyruvate conversion to acetyl-CoA. TCA cycle intermediates, succinate and fumarate, inhibit JMJD-dependent histone demethylation, whereas citrate conversion to acetyl-CoA through ATP citrate lyase (ACLY) and α-KG induce JMJD-dependent histone acetylation and histone demethylation, respectively. Donor molecules and co-factors such as NAD^+, FAD^+ and SAM, that are essential for the activation of histone modifying enzymes are provided through metabolism, ultimately controlling gene expression through the epigenome

Citrate, an intermediate of the TCA cycle, is exported to the cytosol where it participates in fatty acid synthesis, which can be further oxidized to generate acetyl-CoA to boost TCA cycle (Viola et al. 2019). Importantly, it has been shown that IL-4, through AKT/mTORC1 regulates ACLY, the key enzyme that converts citrate and coenzyme A to acetyl-CoA and oxaloacetate, subsequently inducing histone acetylation and M2-associated gene expression in macrophages (Covarrubias and Aksoylar 2016). Nevertheless, additional studies in human macrophages did not support the idea that ACLY regulates the expression of a subset of M2 genes through histone acetylation, and showed that ACLY inhibitors suppress M2 genes via an ACLY-independent manner (Namgaladze et al. 2018). The jmjC family of histone lysine demethylases can remove all three methyl marks using α-KG as substrate, Fe^{2+}, and molecular oxygen as co-factors (Tu et al. 2007). In macrophages, α-KG acts as a cofactor in JMJD3 demethylation function, while accumulation of succinate inhibits its action. Thus, α-KG/succinate ratio controls macrophage polarization since IL-4-induced α-KG accumulation promotes the transcription of M2 genes, while low α-KG/succinate ratio drives M1 polarization. Accordingly, α-KG contributes to endotoxin tolerance and α-KG supplementation in mice reduces septic shock (Liu et al. 2017). Another structurally resembling to α-KG metabolite participating in TCA cycle is fumarate, which acts as an inhibitor of α-KG-dependent demethylating enzymes. Fumarate modifies transcription programs through the induction of H3K4Me3 and H3K27Ac epigenetic marks on the promoters of pro-inflammatory cytokines (Arts et al. 2016).

The metabolite mevalonate, which is part of the HMG-CoA reductase pathway, induces epigenetic changes in macrophages through H3K27Ac on inflammatory gene enhancers and H4K3Me3 enrichment in inflammatory gene promoters through IGF1-R and mTOR activation (Bekkering et al. 2018). In addition to the aforementioned metabolites, SAM serves as a methyl donor for the induction of methyl histone marks (Ye and Tu 2018) (Fig. 2). It is generated by the methionine cycle and although evidence demonstrates a functional correlation between SAM intracellular levels and the induction of DNA/histone methylation, the exact mechanism is not clear. A possible link between these two different functions is through mTOR-dependent serine metabolism, fueling the methionine cycle and resulting in the upregulation of DNA methyltransferases DNMT1 and DNMT3 (Kim 2018).

Although most of the information supporting the effect of AKT on epigenetic regulation of macrophage activation refers to its implication in metabolism, additional mechanisms exist. Protein kinase A (PKA), a member of the same family of kinases as AKT, has been shown to phosphorylate and activate histone demethylase PHF2, allowing it to form a complex with ARID5B (Baba et al. 2011). AKT has also been found to directly phosphorylate histone acetyltransferase p300 and enhance p300 co-transcriptional activity (Huang and Chen 2005), whereas AKT-mediated phosphorylation of EZH2 methyltransferase correlates with reduced H3K27 methylation induction, thus promoting the transcription of previously repressed genes (Cha et al. 2005). Even though the role of AKT on this mechanism has been reported in tumor cells, it is likely that the same events occur in macrophages.

6 Contribution of AKT Kinases to Macrophage Survival and Apoptosis

Apoptosis is the process of programmed cell death. Macrophages are uniquely resistant to pro-apoptotic stimuli, having evolved to function in diverse and hostile microenvironments. Such ability is highly attributed to the PI3K/AKT signaling pathway, the activation of which typically contributes to cell survival.

Macrophages are important players in maintaining or restoring tissue and organismal homeostasis (Mosser et al. 2021). However, under pathological conditions, macrophages can promote a variety of serious pathologies, including insulin resistance, cancer, lung and liver fibrosis, as well as atherosclerosis (Linton et al. 2019; Oishi and Manabe 2016). In fact, macrophages in chronic disease are normally particularly resistant to apoptosis and their prolonged survival is generally associated with disease progression. It is thus of no surprise that most of recent literature on macrophage apoptosis stems from studies of such diseases. Macrophage apoptosis is involved in atherosclerotic plaque formation and factors that inhibit macrophage apoptosis such as LXRa, suppress the disease (Che et al. 2021). Moreover, a number of pathogens have developed intelligent ways to subvert or induce macrophage apoptosis to their advantage (Navarre and Zychlinsky 2000). For example, *Mycobacterium*

tuberculosis suppresses apoptosis in infected macrophages to support intracellular survival (Bade et al. 2021), whereas in viral or bacterial infections macrophages undergo pyroptosis and death (Shotland et al. 2021; Zhang et al. 2021). Non-physiological stimuli such as those initiated by surgical implants may also impair macrophage function and increase apoptotic levels around the foreign material, which may lead to chronic infection and inflammation (Xian et al. 2020). Therefore, it is important to thoroughly decipher the molecular mechanisms by which macrophage survival is tuned to allow targeted induction or inhibition of macrophage apoptosis and manipulate disease pathogenesis or progression.

6.1 AKT Isoforms in Macrophage Survival

In human and murine macrophages, AKT is found constitutively active (phosphorylated) (Babaev et al. 2016a). Generally, suppression of AKT signaling induces apoptosis. The functions of AKT isoforms seem to be redundant regarding macrophage survival. Deletion of either AKT1 or AKT2 in murine macrophages (from $Ldlr^{-/-}$ mice) or AKT3 ablation (from $ApoE^{-/-}$ mice) does not affect cell apoptosis (Ding et al. 2012; Babaev et al. 2014). Moreover, challenge of WT, $Akt1^{-/-}$, $Akt2^{-/-}$, and $Akt3^{-/-}$ peritoneal macrophages with pro-apoptotic stimuli does not reveal any significant variation in apoptotic cell numbers (Babaev et al. 2019). Moreover, deletion of any individual AKT isoform exhibited comparable apoptosis in response to complete IL-3 deprivation in myeloid progenitor cells (Green et al. 2013).

However, when peritoneal macrophages from $Akt1^{-/-}/ApoE^{-/-}$ mice were treated with pro-apoptotic stimuli, they exhibited increased apoptosis compared to control $ApoE^{-/-}$ cells, indicating a possible interplay between AKT1 and ApoE to support macrophage survival (Fernández-Hernando et al. 2007). Furthermore, in idiopathic pulmonary fibrosis (IPF), AKT1-mediated mitophagy rendered alveolar macrophages resistant to apoptosis, whereas deletion of AKT1 resulted in increased apoptotic cell death and protection from the disease (Larson-Casey et al. 2016). In myeloid progenitor cells, AKT1 ablation enhanced apoptosis at low concentrations of IL-3 compared to WT, $Akt2^{-/-}$, and $Akt3^{-/-}$ (Green et al. 2013). These results support the contribution of AKT1 isoform to macrophage survival.

In a recent study, $Ldlr^{-/-}$ mice were reconstituted with hematopoietic cells expressing only one out of the three AKT isoforms, i.e., $Akt1^{only}$ (Akt2/Akt3 double knockout) or $Akt3^{only}$ (Akt1/Akt2 double knockout). Concurrent deficiency of two AKT isoforms led to increased apoptosis of monocytes and macrophages both in normal conditions and under prolonged ER stress. In all cases, $Akt3^{only}$ cells had the most compromised viability, reduced AKT signaling, and displayed an elevated vulnerability to different pro-apoptotic stimuli. Finally, IL-10 was shown to preserve macrophage viability under conditions of ER stress, with $AKT1^{only}$ cells displaying the best survival rates, due to increased IL-10 expression, which suppressed macrophage apoptosis (Babaev et al. 2019). These results are consistent with the idea that AKT1 could be the primary pro-survival isoform in macrophages

(Green et al. 2013; Fernández-Hernando et al. 2007; Liu et al. 2001). The exact molecular mechanism that implicates AKT1 in macrophage survival is yet to be clarified. Mechanistic studies on the impact of AKT in macrophage apoptosis have utilized primarily chemical inhibition or gene silencing, thus the effects may involve more than one AKT isoform, since chemical inhibition is generally not isoform-specific.

6.2 *PI3K/AKT Regulates Macrophage Survival Through Bcl-2 Family Proteins*

It has been suggested that AKT signaling may suppress macrophage apoptosis by maintaining mitochondrial integrity (Duronio 2008). Bcl-2 family of proteins contain Bcl-2 homology (BH) domains and have an important role in the regulation of cell apoptosis by controlling mitochondrial outer membrane permeabilization (MOMP). Bcl-2 proteins are downstream targets of a number of signaling pathways, including PI3K/AKT (Duronio 2008). One of these proteins, Bad, which is a regulator of the caspase-3-mediated apoptosis pathway, is a target of the PI3K/AKT pathway. AKT directly phosphorylates pro-apoptotic Bad at Ser 136 and prevents it from binding to the anti-apoptotic molecule Bcl-X_L, which results in inhibition of the latter (Duronio 2008). Indeed, AKT activation leads to increased Bad phosphorylation and macrophage survival in murine bone marrow-derived macrophages (BMDMs) (Hundal et al. 2001; Rothchild et al. 2016), as well as upregulation of the anti-apoptotic Bcl2 in THP1 cells (Bao and Li 2019). Peritoneal macrophages deficient in EP4 (a receptor of prostaglandin E2, which is increased in activated macrophages) exhibited higher levels of apoptosis when exposed to pro-apoptotic stimuli, an effect partially due to inhibition of AKT and Bad phosphorylation (Babaev et al. 2008). In other studies, prolonged ER stress decreased AKT and Bad phosphorylation levels and impaired cell survival (Babaev et al. 2019, 2016b). *Mycobacterium tuberculosis*, an intracellular pathogen with macrophage tropism, promoted macrophage survival to its advantage through AKT-mediated Bad phosphorylation (Maiti et al. 2001). Therefore, PI3K/AKT signaling may regulate macrophage apoptosis through direct phosphorylation of Bad at Ser 136.

Myeloid leukemia cell differentiation protein (Mcl-1) is also a member of the Bcl-2 family and seems to be involved in macrophage survival. An early study suggested that constitutive activation of AKT1 regulates macrophage survival through Mcl-1 in human monocyte-derived macrophages and RAW 264.7 cells (Liu et al. 2001). In another study, activation of AKT signaling in synovial macrophages from rheumatoid arthritis (RA) patients were shown to confer resistance to apoptosis through upregulation of Mcl-1 ex vivo, whereas inhibition of the PI3K/AKT pathway significantly reduced Mcl-1 and increased apoptotic cell death (Liu et al. 2006).

GSK3 phosphorylates Mcl-1, leading to its degradation, and AKT has been shown to inhibit GSK3. Typically, GSK3 activation augments cell vulnerability to apoptotic stimuli (Opferman 2006). Indeed, the intracellular parasite Leishmania dovonavi,

inhibited host cell apoptosis in RAW 264.7 cells and BMDMs to its advantage by AKT-mediated regulation of the GSK3β/β-catenin/FOXO1 axis. Specifically, AKT activated the anti-apoptotic β-catenin through phosphorylation of GSK3β, in a way that the latter could no longer sequester the former (Gupta et al. 2016). In the same study, AKT also inhibited the pro-apoptotic transcription factor FOXO1 (Gupta et al. 2016). Forkhead transcription factors have been shown to be phosphorylated by AKT in other cell types, consequently leading to their sequestration and degradation in the cytoplasm, which results in downregulation of a number of pro-apoptotic factors, such as Fas death receptor ligand (FasL) or the Bcl-2 family protein Bim (Duronio 2008). Thus, AKT signaling may affect macrophage survival by controlling the Bcl-2 family of proteins in ways that include direct activation through phosphorylation, or phosphorylation and subsequent inhibition of intermediate regulatory molecules such as GSK3 and FOXO1.

6.3 AKT Promotes Macrophage Survival Through NFκB and mTORC2

Transcription factor NFκB supports cell survival by regulating several pro-survival genes, such as the anti-apoptotic Bcl-2 family of proteins, Bcl-2 and Bcl-X_L. NF-κB activation is controlled by IκB, which in resting cells is bound to NFκB, maintaining it in an inactive state. IκB Kinase (IKK)α and IKKβ, which form a complex, phosphorylate IκB resulting in IκB degradation and NFκB release. AKT can activate IKKα and, in turn, activate NFκB and promote cell survival (Duronio 2008). In EP4-deficient murine macrophages, decreased AKT phosphorylation was accompanied by downregulation of NFκB target genes, indicating that the lack of EP4 negatively affected macrophage survival through suppression of PI3K/AKT and NFκB pathways (Babaev et al. 2008). Moreover, it has been observed that oxLDL caused a dose-dependent inhibition of apoptosis in cultured BMDMs through AKT activation, which resulted in phosphorylation of IκBα and Bad (Hundal et al. 2001). Therefore, AKT signaling may promote macrophage survival by inducing a subset of anti-apoptotic genes through the NFκB pathway.

IKK induces AKT Ser 473 phosphorylation through mTORC2. Pharmacological inhibition and knockdown of IKK, as well as kinase-dead IKK expression, suppressed mTORC2 activity and reduced AKT Ser 473 phosphorylation in different cell lines (Xu et al. 2013b). Indeed, it was demonstrated that IKKα deficiency in mouse macrophages blocks mTORC2-mediated AKT phosphorylation at Ser 473, rendering cells highly susceptible to pro-apoptotic stimuli in vitro (Babaev et al. 2016a). Therefore, IKKα deficiency promotes macrophage apoptosis through suppression of AKT signaling.

Rictor is a necessary component of mTORC2, which directly induces AKT Ser 473 phosphorylation. Murine bone marrow monocytes and peritoneal macrophages deficient in Rictor exhibited both decreased mTORC2 signaling and reduced AKT

Ser 473 phosphorylation, being sensitive to pro-apoptotic stimuli (Babaev et al. 2018). Thus, IKKα and mTORC2 may promote macrophage survival through AKT phosphorylation.

6.4 P38 MAPK and JNK Affect Macrophage Survival Through AKT Signaling

P38 MAPKs respond to a wide variety of stress stimuli and are involved, among others, in cell survival. Activation of p38 MAPK can have pro- or anti-apoptotic effects depending on the cellular environment. A study using BMDMs and human peripheral monocytes showed that ROS generated from the NADPH oxidase complex participates in the M-CSF-induced suppression of apoptosis by regulating AKT and p38 MAPK (Wang et al. 2007). A subsequent study demonstrated that ER stress-induced activation of p38 suppresses macrophage apoptosis through AKT signaling (Seimon et al. 2009).

JNKs also belong to the MAPK family, and regulate apoptosis partly via PI3K/AKT (Manning and Cantley 2007). Recent evidence demonstrated that JNK1 signaling antagonizes AKT activity in murine peritoneal macrophages (Babaev et al. 2016b). Specifically, sustained JNK signaling induced by ER stress elevated macrophage apoptosis by decreasing AKT Ser 473 and Bad Ser 136 phosphorylation. Consistent with a study showing that JNK regulates p-AKT via PTEN (Vivanco et al. 2007), genetic deficiency and chemical inhibition of PTEN nearly reversed JNK-mediated suppression of AKT phosphorylation during ER stress (Babaev et al. 2016b), thus affecting apoptosis. An overview of the contribution of AKT signaling in macrophage survival and apoptosis is shown in Fig. 3.

6.5 MiRs Regulate AKT-Mediated Macrophage Apoptosis

MiRs have been also found to affect the viability of cells, including macrophages, by regulating different signaling pathways, such as the PI3K/AKT. Recent evidence suggests that miR-155 inhibits oxLDL-induced macrophage apoptosis, at least partially, by downregulating the expression of p85a (a negative regulator of the PI3K/AKT pathway), thus preventing AKT deactivation in RAW 264.7 cells (Ruan et al. 2020). Moreover, SHIP1, which suppresses PI3K/AKT signaling, has been shown to be targeted by miR-155 (O'Connell et al. 2009). Indeed, in *miR155*$^{-/-}$ macrophages, SHIP1 protein levels were increased, whereas AKT phosphorylation was suppressed, and that was associated with reduced Bad phosphorylation and increased apoptosis (Rothchild et al. 2016). Another study using THP-1 cells showed that miR-32 downregulated PTEN, thereby activating PI3K/AKT signaling

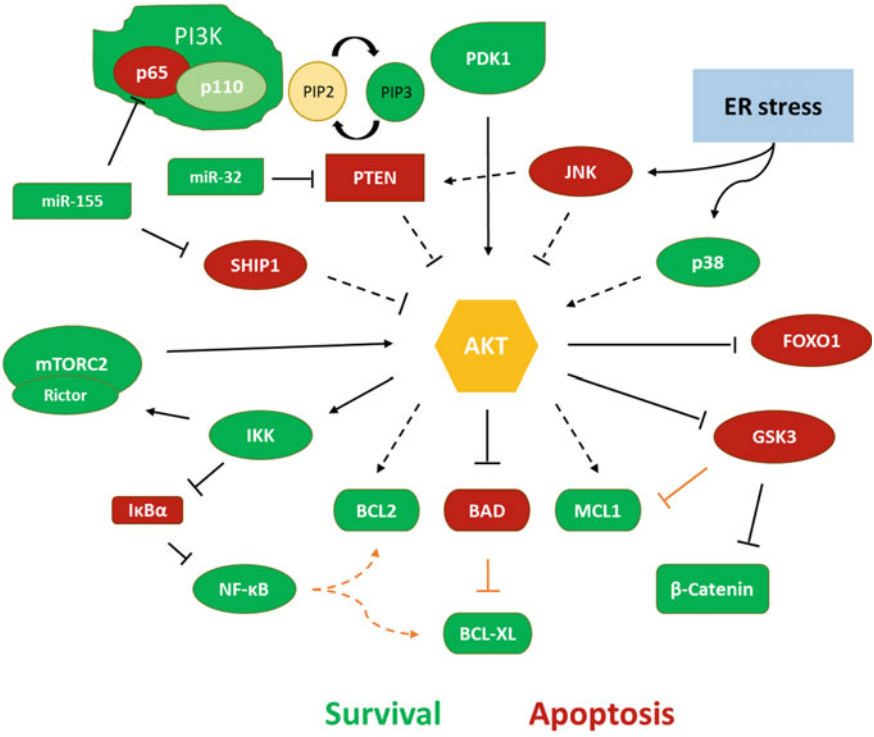

Fig. 3 AKT signaling in macrophage survival and apoptosis. PI3K induces the production of PIP2 and PIP3, which leads to the translocation of AKT and Phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane. PDK1 phosphorylates AKT, resulting in its partial activation. mTORC2 also phosphorylates AKT on another residue, fully activating the latter. PTEN works by dephosphorylating PIP3 to PIP2, terminating PI3K signaling. Factors in red promote apoptosis, whereas those in green promote survival. Black arrows are used when at least one publication has shown the indicated interaction specifically in macrophages. Orange arrows indicate possible interactions, which have been demonstrated in other cell types but not yet shown in macrophages. Dashed arrows indicate indirect interactions

pathway, which in turn led to downregulation and upregulation of pro- and anti-apoptotic proteins, respectively, thereby attenuating apoptosis of the aforementioned cells (Bao and Li 2019).

7 Conclusions

Since the discovery of AKT kinase isoforms, a large amount of information is available on the role of these signaling molecules in cell homeostasis and function. Generation of isoform-specific knockout animals and isoform-specific antibodies has allowed us to decipher distinct functions for each AKT isoform. Important

roles for AKT kinases have been described in macrophages, which include regulation of TLR signaling, cytokine signaling, phagocytosis and pathogen elimination, cell metabolism, and more. Yet, the redundant and non-redundant functions of each isoform have not been fully characterized, and remain an important challenge for therapeutic targeting of this kinase family in inflammatory diseases and cancer.

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Control of CD4$^+$ T Cell Differentiation and Function by PI3K Isoforms

Benjamin Cameron, Syed Aymaan Zaheer, and Margarita Dominguez-Villar

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Abstract The phosphoinositide-3-kinase (PI3K) pathway is a highly conserved intracellular signaling pathway involving numerous key effectors which, in response to diverse extracellular stimuli, modulate the phenotype and function of most mammalian cell types in a pleiotropic manner. PI3K signaling plays a critical role in the development, activation, and differentiation of lymphocytes. In particular, the PI3Kδ and PI3Kγ isoforms have been shown to carry out essential, non-redundant roles in T cells, and therefore, tight regulation of the PI3K pathway is important to maintain the balance between immune tolerance and inflammation. Recent and ongoing efforts to manipulate the biology of T helper cell subsets in the treatment of autoimmune conditions, inflammatory disorders, as well as cancer have shown promising results, and targeting the PI3K pathway may be beneficial in these contexts. However, more insight as to the precise function of individual PI3K isoforms in pathogenic and protective immune cell subsets is still required, and how exactly PI3K signaling is regulated and integrated with classical immune pathways. This chapter provides an overview of the role of PI3K isoforms in the differentiation and

B. Cameron and S. A. Zaheer—These authors contributed equally to this manuscript.

B. Cameron · S. A. Zaheer · M. Dominguez-Villar (✉)
Faculty of Medicine, Imperial College London, London W2 1PG, UK
e-mail: m.dominguez-villar@imperial.ac.uk

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function of T helper cell subsets, within the broader context of targeting this pathway to potentially alleviate immunopathology.

1 Introduction

Since their discovery in the late 1980s, PI3Ks have been extensively studied for their role in growth-factor receptor signaling (Hawkins et al. 2006), and hyperactivation of the PI3K pathway is considered a hallmark of cancer and tumorigenesis (Vogt et al. 2010; Fresno Vara et al. 2004). In mammals, there are eight isoforms of PI3K divided into three classes (class I–III) based on their sequence homology, structure, regulation, and lipid substrate specificity (Fruman et al. 2017). Advancements in the development of gene-targeted mice and isoform-selective small molecule inhibitors have been instrumental in expanding the framework of PI3K signaling and have uncovered essential roles for PI3Ks in most cell types (Okkenhaug 2013). Within the PI3K family, the best studied are class IA and class IB PI3Ks, whereas class II and III PI3Ks are less well-understood (Vanhaesebroeck et al. 2005) and will not be discussed hereafter.

Expression of the catalytic subunits p110δ (class IA) and p110γ (class IB) is restricted to leukocytes, and this fact provides the rationale for widespread research into the function of PI3Kδ and PI3Kγ in the immune system (Ghigo et al. 2010; Swat et al. 2006; Vanhaesebroeck et al. 2010). Indeed, it has been shown that PI3Kδ and PI3Kγ carry out distinct, non-redundant roles in both innate and adaptive immunity (Okkenhaug 2013; Koyasu 2003), and understanding differential PI3K signaling and isoform selectivity has become considerably important. Diverse aspects of T cell biology spanning from their development in the thymus to their activation and function in the periphery involve PI3K signaling (Okkenhaug 2013).

Classically, receptor tyrosine kinases (RTKs) activate class I PI3Ks, whereas class IB PI3Ks are activated by G protein-coupled receptors (GPCRs) (Fruman and Bismuth 2009; Han et al. 2012). PI3Kδ plays a unique role in signal transduction downstream of antigen and cytokine receptors, while PI3Kγ is important in chemokine-dependent processes (Hawkins and Stephens 2015). Not surprisingly, PI3Kδ and PI3Kγ have emerged as critical players in the differentiation and function of $CD4^+$ T cells (Okkenhaug 2013). Exactly how the activity of these isoforms is integrated with canonical immune regulatory pathways is not well understood. Dysregulation of the PI3K pathway has been implicated in T cell-mediated autoimmune and inflammatory disorders (Ghigo et al. 2010; Chen et al. 2018; Way et al. 2016; So and Fruman 2012). Current studies aim to understand how PI3K integrates immune and non-immune cues, whether inputs function redundantly or additively and if specific isoforms are preferentially activated by distinct stimuli. Such mechanistic insights will better inform the development and use of PI3K inhibitors for immune-related diseases as well as cancer, where targeted manipulation of T cell subsets has become an exciting approach (So and Fruman 2012; Stark et al. 2015;

Wymann 2012). This chapter will focus on the role of the PI3K pathway in the differentiation and function of CD4+ T helper (Th) cells.

2 Lessons Learned from Patients with Immunodeficiencies Due to Mutations on PI3K Subunits

While mouse models with deletion of specific PI3K isoforms have provided relevant data on the function of PI3K isoforms in immune cell function and their involvement in disease, monogenic human diseases where mutations in specific PI3K isoforms confer increased or decreased cell function have provided invaluable information on the consequences of dysfunctional PI3K isoforms in humans. For example, activated phosphoinositide-3-kinase-δ syndrome (APDS) is an immunodeficiency characterized by either a gain-of-function mutation in the *PIK3D* gene, resulting in mutations in p110δ (APDS1), or a loss-of-function mutation in the gene *PIK3R1*, leading to mutations in the p85 regulatory subunit of PI3K (APDS2) (Dornan et al. 2017; Lucas et al. 2016). Both forms of APDS result in hyperactive p110δ signaling, typically leading to recurrent respiratory infections often accompanied by bronchiectasis and ear and sinus damage. The adaptive immune compartment in these patients is characterized by increases in the frequency of transitional B cells and decreases in differentiated and class-switched B cells (Avery et al. 2018). T cells show impaired function and increased tendency to senescence, and the patients display global lymphopenia (Lucas et al. 2016; Preite et al. 2018, 2019). Furthermore, patients frequently present with neurodevelopmental delay (Michalovich and Nejentsev 2018). Despite the fact that the mutations in APDS2 are located in p85, which is present in several class IA PI3Ks besides p110δ, APDS2 largely phenocopies APDS1 (Dornan et al. 2017).

APDS causes numerous defects on CD4+ T cells, including elevated numbers of circulating T follicular helper (cTfh) cells (Preite et al. 2018; Bier et al. 2019), which are skewed toward a Th1-like phenotype characterized by the upregulation of the transcription factor Tbet and increased secretion of IFNγ (Bier et al. 2019). In addition, an increase in the secretion of Th2 type cytokines including IL-4, IL-5, and IL-13 by naïve, Tfh, and memory CD4+ T cells from APDS patients has been reported (Bier et al. 2019). In vitro, naïve CD4+ T cells from APDS1 patients display impaired differentiation into IL-21-secreting Tfh cells (Bier et al. 2019). Reduced numbers of total CD4+ T cells have been also reported from a cohort of APDS1 patients, along with decreased frequency of peripheral naïve T cells, and higher numbers of effector memory T cells (Lucas et al. 2014). However, lymphopenia is not observed in all patients with APDS, and in fact, normal numbers of total CD4+ T cells, and higher numbers of total Tfh cells, have been reported in an APDS2 patient, while germinal center (GC) Tfh cells were shown to be reduced, suggesting that p85 is important in Tfh cell maturation (Fonte et al. 2016). Low numbers of recent thymic immigrant (RTE) cells have been reported in an APDS1 patient, indicating impaired regeneration of naive CD4+ T cells (Goto et al. 2017).

Since the first case was published in 2013, over 200 APDS patients have been identified worldwide, and treatment options mainly include immunoglobulin treatment and prophylactic antibiotics (Michalovich and Nejentsev 2018). Hematopoietic stem cell transplantation (HSCT) results in a high thirty-year survival rate and reduction in symptoms, but graft failure, lymphoproliferative disease, and autoimmune cytopenia are common effects after transplant in APSD1 (Okano et al. 2019). Successful HSCT examined after five (Elkaim et al. 2016) and nine months (Kuhlen et al. 2016) has been reported in APSD2 patients. Treatment with the mTOR inhibitor rapamycin improved T cell function and cell counts in APSD1 patients (Lucas et al. 2014), and showed improved clearance of infection and reductions in tonsil, liver, spleen, and lymph node swelling in APSD2 patients (Elkaim et al. 2016). A 12-week, open-label, dose-escalation clinical trial examining the effect of the p110δ inhibitor leniolisib in six APDS1 patients has shown marked improvement in patient prognosis, including reductions in the frequency of chronically activated/exhausted $CD4^+$ T cells (Rao et al. 2017). Two more clinical trials for p110δ inhibitors in APDS, sponsored by Novartis (NCT02435173) and GlaxoSmithKline (NCT02593539), are currently underway.

Several patients with loss-of-function mutations in the *PIK3D* gene have been identified (Sogkas et al. 2018). The condition is characterized by a near-total loss of B cells, with one of the examined patients also displaying T cell lymphopenia with a reduction in the numbers of memory T cells and a loss of perforin and granzyme expression by NK cells (Lucas et al. 2016).

3 T Cell Activation, Proliferation, and Survival

PI3K signaling is necessary for activation of primary T cells from healthy individuals (Kane and Weiss 2003), and it contributes to the activation of important transcription factors that mediate T cell function, including nuclear factor of activated T cells (NFAT) and extracellular signal-regulated kinases (ERK) 1/2 (Courtney et al. 2018; Hermann-Kleiter and Baier 2010). In agreement with the role of PI3K in promoting T cell activation, the phosphatase PTEN (Hawse et al. 2015) and transmembrane inhibitor of PI3K (TrlP) (Uche et al. 2018) which prevents the activation of the p110 subunit by the p85 subunit (DeFrances et al. 2012), have been also shown to be downregulated during T cell activation.

Survival and differentiation of T cells are influenced by downstream effectors of PI3K. In conventional $CD4^+$ T cells (Tconv), inhibition of all PI3K isoforms significantly reduces proliferation in both mouse (Hu et al. 2019) and human cells (Blanco et al. 2015). With regards to the specific roles of class I PI3Ks, studies suggest that inhibition of the p110α, p110β, and p110γ isoforms individually have little to no effect on Tconv proliferation (Blanco et al. 2015; Ahmad et al. 2017). Conversely, there are contrasting data on the role of p110δ in Tconv proliferation. Thus, while inhibition of p110δ in vitro by CAL-101 on both healthy human and mouse Tconv and a p110δ inactivating mutation in mouse Tconvs have no effects

on T cell proliferation (Ahmad et al. 2017), significant decreases in proliferation of human T cells after p110δ inhibition by IC-87114 has been demonstrated in vitro (Blanco et al. 2015). Furthermore, inhibition of p110δ by IC-87114 reduces human and mouse CD4+ T cell proliferation to a similar extent as simultaneous inhibition of all isoforms (Soond et al. 2010). In the latter study, p110δ deletion in mice also reduced proliferation of naïve CD4+ T cells (Soond et al. 2010).

4 T Helper (Th) Cell Generation and Maintenance

CD4+ T helper (Th) cells are key orchestrators of adaptive immune responses, and the diverse functionality of these cells relies on differentiation into distinct effector subsets. Naïve T cell activation occurs via T cell receptor (TCR) recognition of an antigenic peptide complexed to an MHC class II molecule and the interaction of the CD28 co-receptor with costimulatory molecules CD80 and CD86 expressed on the surface of antigen-presenting cells (APCs). Depending on the nature of the pathogen, the innate immune response generates the cytokine milieu which critically shapes T cell fate. Th cell differentiation is tightly controlled and involves dramatic changes to the cell transcriptome and proteome, ultimately giving rise to lineage-specific gene-regulatory programs which confer specific effector function. In conjunction with cytokine-receptor JAK/STAT pathways, the precise qualitative and quantitative signaling from the TCR/CD28 complex and other co-receptors, as well as signal integrations and crosstalk between these networks skew T cell fate.

PI3Ks are stimulated by many of the fate-deciding receptor-ligand interactions in T cell-dependent processes, and evidence shows that pan-inhibition of class I PI3Ks significantly impairs differentiation along Th lineages (Okkenhaug 2013; So and Fruman 2012; Okkenhaug and Vanhaesebroeck 2003) (Fig. 1). In fact, the relative activation of the PI3K pathway appears to play an important role in maintaining immunological tolerance (Han et al. 2012). While evidence suggests p110δ and p110γ are preferentially recruited to the immune synapse (Kok et al. 2009), p110α and p110β together account for up to 50% of total PI3K activity in T cells (So and Fruman 2012) and their precise role in T cell-dependent responses is still being investigated. Manipulating PI3Kα and PI3Kβ through genetic approaches often results in embryonic lethality, yet in vitro experiments using selective inhibitors are beginning to provide insight into the functions of these isoforms in T cells (Okkenhaug 2013; Okkenhaug and Vanhaesebroeck 2003). Collectively, seminal studies have demonstrated that optimal T cell activation and antigen-dependent responses require the activity of both PI3Kδ and PI3Kγ (Deane and Fruman 2004).

In recent years, selective PI3K inhibitors have been developed with the goal of alleviating autoimmune and inflammatory disorders driven by aberrant Th cell responses. Several selective and dual PI3Kδ and PI3Kγ inhibitors are in clinical trials and many more in pre-clinical development (Stark et al. 2015). These small molecules offer greater specificity, decreased toxicity, and exhibit superior pharmacodynamics compared to pan-PI3K inhibitors (So and Fruman 2012; Wang et al.

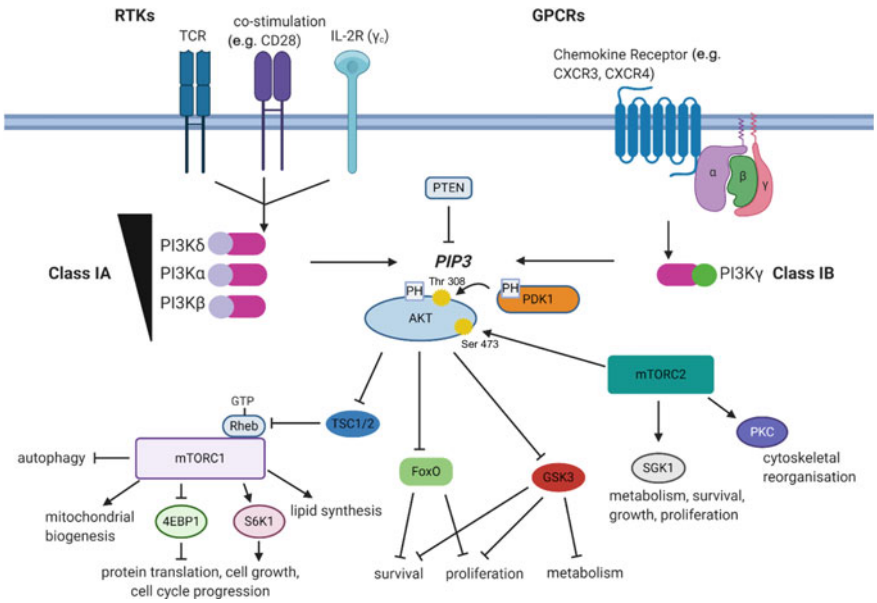

Fig. 1 Regulation and function of the PI3K pathway in T cells. Class IA and class IB PI3Ks are activated by RTKs and GPCRs, respectively. PI3Kδ (and PI3Kγ) are the predominant isoforms in T cells. In addition to immune signals (e.g., antigen, co-stimulation, cytokines, chemokines), environmental cues (e.g., growth factors) and nutrients (e.g., amino acids, glucose) can engage class I PI3Ks and trigger the generation of PIP3. PIP3 recruits PDK1 and AKT via their PH domain, promoting PDK1- and mTORC2-dependent activation of AKT, which acts on a variety of downstream effectors (not all pictured) including the tuberous sclerosis 1 (TSC1)-TSC2 complex to activate mTORC1. AKT/mTORC1-dependent pathways promote cell survival, cell cycle progression, growth, proliferation, and metabolic reprogramming. The mTORC2 effector SGK1 has similar effects while PKC is important in mediating cytoskeletal rearrangement and cell motility

2015). Perhaps as a result of the pleiotropic effects of PI3K signaling, some studies have uncovered conflicting results underscoring the need to delineate differential regulation of this pathway in pathological and protective immune cell subsets. To this end, elucidating the molecular mechanisms by which PI3K isoforms control the generation and function of Th cells is vital. The following sections will discuss current knowledge of PI3K isoforms in Th1, Th2, Th9, and Th17 cell differentiation and function, and will highlight important effectors of the pathway which are involved in this context.

4.1 Th1 Cells

Th1 responses enhance cytotoxic T lymphocyte (CTL) function and stimulate macrophages and neutrophils to promote clearance of intracellular pathogens (Ruterbusch et al. 2020). Generation of Th1 cells requires IL-12 and IFN-γ, which drive STAT4- and STAT1-dependent upregulation of the Th1 master regulator Tbet and epigenetic remodeling at the Th1 *loci*. Tbet potentiates IFN-γ production and suppresses alternative T cell fates (Jenner et al. 2009). Overactive or misdirected Th1 responses are associated with autoimmune diseases including multiple sclerosis (MS), type 1 diabetes (T1D), and rheumatoid arthritis (RA) (Dardalhon et al. 2008).

Impaired differentiation along the Th1 and Th2 lineages is observed in $p110\delta^{D910A/D910A}$ mice, which contain a point mutation that induces expression of a catalytically inactive form of p110δ (Vanhaesebroeck et al. 2005). Antigen-stimulated $p110\delta^{D910A/D910A}$ T cells show significantly impaired proliferation and a reduced number of IFNγ-producing cells in vivo compared to wild type T cells (Okkenhaug et al. 2006). In fact, sustained PI3Kδ activity has been shown to drive IFNγ production, and inhibition of PI3Kδ potently blocks Th1-like effector and memory responses in both mice and humans (Soond et al. 2010). In vitro, PI3Kδ controls clonal expansion in a manner dependent on association with the TCR/CD28 signaling complex and γ-chain cytokines (including IL-2) and IL-12 (Okkenhaug et al. 2006). PI3Kδ promotes AKT-dependent phosphorylation of FoxO1/3a, sequestering these proteins from the nucleus (Luo and Li 2018). FoxO regulate T cell quiescence, trafficking, survival of naïve and memory T cells, and typically antagonizes cellular differentiation (Lin et al. 2004).

Th1 cells undergo extensive metabolic reprogramming to support rapid proliferation and cytokine production. Induction of aerobic glycolysis in what is referred to as the "Warburg Effect" is critical, and failure to upregulate glucose metabolism leads to profound proliferative defects, decreased cytokine production, and can promote apoptosis (Gerriets and Rathmell 2012). CD28 provides input to PI3Kδ to coordinate metabolic reprogramming upon T cell activation by activating AKT/mTOR, which strongly enhances glucose uptake, amino acid, and lipid metabolism (Salmond 2018). In Th1 inducing conditions, limiting the availability of glucose upon activation significantly impairs proliferation, IFNγ production, and results in lower levels of Tbet-expressing cells (Jacobs et al. 2008). Pan-inhibition of PI3K via LY294002 abrogates the typical increase in glucose uptake upon T cell activation (Frauwirth et al. 2002), and T cells expressing a constitutively active transgene of AKT show increased glucose uptake, largely independent of CD28 (Jacobs et al. 2008). Some metabolic enzymes also play a direct role in Th1 differentiation, such as glyceraldehyde 3-phosphate hydrogenase (GAPDH), for example, whereby induction of glycolysis sequesters the ability of GAPDH to bind the *Ifng* 3' UTR and enhances the translation rate of IFNγ (Peng et al. 2016).

Downstream of PI3K, mammalian target of rapamycin (mTOR) integrates immune signals, environmental cues, and nutrient sensing to dictate T cell fate and function (Huang et al. 2020). mTOR exists in two separate complexes, mTORC1,

and mTORC2, which differ in their molecular composition, regulation, function, and have recently been shown to differentially regulate T cell fate (Chornoguz et al. 2017; Delgoffe et al. 2011; Lee et al. 2010; Wang et al. 2020). mTORC1 activity is vital for Th1 cells, and CD28 and IL-2R promote PI3Kδ-mediated activation of this complex, while IL-12 and IFN-γ also drive its sustained activation (Powell and Delgoffe 2010). T cells from mice bred with a floxed mTOR gene-specific for *Vaccinia* virus, a strong inducer of Th1 responses, were unable to differentiate into Th1 effector cells in vivo and mTOR deficiency results in failure to upregulate Tbet in Th1-skewing conditions (Delgoffe et al. 2009). In mice, mTORC1 was shown to control the phosphorylation of Tbet and enhance its transcriptional activity at the *Ifng* gene *locus* (Chornoguz et al. 2017). Crosstalk between mTOR and STAT/SOCS signaling networks takes place whereby mTORC1 enhances IL-12 responsiveness via inhibition of SOCS3, promoting STAT4-dependent gene transcription (Saleiro and Platanias 2015). Consistent with this, *Rheb*$^{-/-}$ (mTORC1-deficient) T cells show increased expression of SOCS3, and SOCS3 silencing restores Th1 differentiation (Powell and Delgoffe 2010). Further, IL-12-induced STAT4 activation is reduced in mTOR-deficient T cells (Delgoffe et al. 2009).

Inhibitors of PI3Kγ attenuate Th1 differentiation and reduce cytokine production, though not as significantly as PI3Kδ inhibitors (So and Fruman 2012). The molecular mechanisms linking PI3Kγ to the events during T cell activation and polarization are not well understood (Alcazar et al. 2007). Links between p110γ and TCR signaling have been evidenced and involve indirect association with Lck and ZAP70 via GTPase adaptor proteins (So and Fruman 2012). Indeed, TCR-dependent signaling is marginally impaired in p110γ$^{-/-}$ cells (Alcazar et al. 2007). TCR-induced activation of the GTPase Rac regulates actin polymerization and promotes APC-conjugate formation, and PI3Kγ has been shown to mediate this process (Alcazar et al. 2007). In experimental autoimmune encephalomyelitis (EAE), the commonly employed rodent model for multiple sclerosis (MS), mice with genetic ablation of p110γ (*Pik3cg*$^{-/-}$) show significantly reduced signs of clinical disease including the absence of characteristic lesions and central nervous system (CNS) demyelination. Moreover, the priming of pathogenic CD4$^+$ T cells is severely diminished, a result of both suboptimal dendritic cell activity and T cell-intrinsic defects. As a result, these T cells were unable to acquire the surface phenotype, trafficking capability, and proliferative capacity of encephalitogenic CD4$^+$ T cells and produced less IFNγ (Comerford et al. 2012). In non-obese diabetic (NOD) mice, the animal model of T1D, activation of autoreactive effector T cells was suppressed and Th1-associated cytokine production reduced by the PI3Kγ inhibitor AS605240 both in vitro and in vivo, results that encourage further investigation into targeting PI3Kγ in T1D (Azzi et al. 2012). Collectively, PI3Kγ appears to be important in inflammatory cell priming and recruitment and further investigation is required to understand the role of this isoform in organ-specific autoimmune diseases (Comerford et al. 2012).

While most studies have focused on PI3Kδ and PI3Kγ T cell-dependent responses, the contribution of p110α and p110β has not been determined unequivocally. Recently, PI3Kα has been implicated in the events taking place during early T cell

activation, and p110$^{-/-}$ T cells differentiated in vitro show enhanced effector function marked by higher Tbet expression and IFNγ production (Aragoneses-Fenoll et al. 2018). The study suggests that p110α may compete with and downmodulate p110δ signaling, implying that the increased effector function of p110α$^{-/-}$ T cells is due to more robust activation of PI3Kδ. If true, such findings could have implications in anti-tumor immunity. Additional studies are required to determine the role of PI3Kα (and PI3Kβ) in antigen-dependent T cell signaling.

In summary, PI3Kδ is critical for the generation of Th1 cells and although PI3Kγ perhaps plays a lesser role, it is involved in priming and migration to sites of inflammation. In line with these findings, PI3Kδ and PI3Kγ have become clinically relevant targets for treatment of Th1-mediated autoimmune and inflammatory disorders where the goal is to reduce the generation of harmful T cell subsets and production of damaging cytokines. Future studies will help to determine whether PI3Kα and PI3Kβ function redundantly or have distinct roles in Th1 cell generation and function.

4.2 Th2 Cells

Extracellular parasites including helminths trigger Th2 responses which stimulate eosinophils, basophils, and mast cells and promote B cell IgE antibody class-switching to facilitate tissue repair and regeneration (Nakayama et al. 2017). Generation of Th2 cells requires IL-4 which promotes STAT6-dependent upregulation of the Th2 master regulator, GATA-3. Th2 cells secrete IL-4, IL-5, and IL-13 (Zhang et al. 2014), and aberrant Th2 responses contribute to chronic inflammatory diseases including asthma and allergy (Ruterbusch et al. 2020; Nakayama et al. 2017).

Pan-inhibitors of PI3K reduce Th2-mediated airway inflammation in mouse models of allergic asthma (Nashed et al. 2007), and class I PI3Ks have since been implicated in nearly all aspects of asthma pathophysiology (Takeda et al. 2010). P110δ$^{D910A/D910A}$ mice exhibit impaired Th2 differentiation and cytokine production and show protection from Th2-mediated inflammation in an ovalbumin-dependent airway hypersensitivity model (Nashed et al. 2007). The TCR/CD28 complex, IL-2R, IL-4R, and inducible costimulatory signal OX40 promote Th2 differentiation via PI3Kδ-dependent mechanisms (Han et al. 2012). In mice, mTOR has been shown to enhance the translation rate of GATA-3 in a PI3Kδ-dependent manner. Naïve T cells from transgenic mice stimulated in Th2 conditions using OVA show decreased GATA-3 protein levels when treated with LY294002 despite any observable changes in mRNA levels (Cook and Miller 2010). These results are reproduced upon rapamycin (mTOR inhibition) treatment of T cells prior to stimulation, further confirming that PI3Kδ-mediated translational control of GATA-3 occurs via mTOR.

Costimulatory interactions play an important role in modulating PI3K activity in Th2 cells. Mice deficient in OX40 or OX40L exhibit drastically reduced Th2 lung inflammation in an ovalbumin-dependent model of allergic asthma, and blocking OX40L is effective in suppressing memory T cells and Th2 cytokine production

(Hoshino et al. 2003). OX40 promotes PI3Kδ-mediated, NFAT-dependent transcription of *Il4* (Croft et al. 2009). Mouse and human genetic analyses have implicated T cell immunoglobulin and mucin domain 1 (TIM-1) in airway hyperresponsiveness, Th2 responses, and atopic disease (Binne et al. 2007), and it has been shown to associate with the TCR complex and induce physical interaction between IL-2 inducible T cell kinase (ITK) and PI3Kδ (Binne et al. 2007). Notably, GATA-3 promotes ITK expression during Th2 commitment in mice, and *Itk*-deficient mice exhibit poor Th2 responses and decreased allergic airway inflammation (Miller et al. 2004; Schwartzberg et al. 2005).

In contrast to Th1 cells which primarily rely on mTORC1 activity, mTORC2 has been shown to be indispensable for Th2 cell differentiation (Han et al. 2012; Delgoffe et al. 2011; Lee et al. 2010). mTORC1 is important in early stages of Th2 differentiation and glycolysis inhibition has been shown to reduce IL-5 and IL-13 in vitro and in vivo (Stark et al. 2019). mTORC2 participates in crosstalk with STAT/SOCS networks in Th2 cells by inhibiting SOCS5, which promotes IL-4 responsiveness and increased STAT6 dependent gene transcription (Delgoffe et al. 2011; Saleiro and Platanias 2015). mTORC2 effectors protein kinase C (PKC-Θ) and serine/threonine-protein kinase 1 (SGK1) cooperate with signals from TCR/CD28 to drive activation of nuclear factor kappa B (NF-ĸB), NFAT, and activator protein 1 (AP-1), key transcription factors that promote expression of IL-4 and GATA-3 (Oh and Ghosh 2013). SGK1 represses IFNγ production and prevents degradation of JunB, an important regulator of IL-4 production (Heikamp et al. 2014). Mice with a T cell-specific deletion of SGK1 showed resistance from allergic asthma due to diminished Th2 responses (Heikamp et al. 2014). T cells deficient in Rictor, the key subunit of mTORC2, exhibit a profound decrease in Th2 differentiation accompanied by attenuated TCR-induced proliferation. In studies of mTORC2-deficient mutant cell lines, complementation with activated PKC-Θ but not AKT was able to revert GATA-3 expression and restore Th2 defects (Lee et al. 2010). These results suggest that mTORC2 serves as a linker between TCR/CD28, PKC-Θ, and PI3K signaling. In contrast to mTORC1, the upstream regulation of mTORC2 has not been well established, and whether PI3K isoforms control mTORC2 activity is unknown. Interestingly, the mTORC2 subunit mSin1 contains a PH domain that has been shown to bind PIP3 (Yuan and Guan 2015).

Evidence that class I PI3K contributes to Th2-mediated inflammatory diseases has sparked interest in selectively targeting PI3Kδ to attenuate aberrant Th2 responses. However, exactly how PI3K isoforms control Th2 polarization and cytokine production at the molecular level is not well described. It will be important to uncover how PI3K integrates Th1 versus Th2 skewing signals and how mTOR differentially regulates T cell fate, specifically whether PI3K isoforms control mTORC2 activity and whether this axis can be discretely targeted.

4.3 Th9 Cells

The Th9 lineage has recently been described in mice and humans as IL-9-producing CD4+ T cells that require TGF-β and IL-4 for their generation in vitro. Though there are a number of transcription factors contributing to Th9 cell differentiation, PU.1, IRF4, and STAT6 appear to be the most important factors in driving IL-9 production, a pleiotropic cytokine with a number of implications in human disease (Kaplan 2013). Primarily, Th9 cells play a role in helminth immunity (Licona-Limon et al. 2017; Pinto and Licona-Limon 2017), are associated with anti-tumor responses (He et al. 2020), and also participate in allergic inflammation and autoimmune diseases (Jia and Wu 2014). Although few studies have directly studied PI3K isoforms in the generation of Th9 cells, there are a few distinctive points worth discussing (Wang et al. 2020).

In contrast to Th1 and Th2 cells, in which inhibition of PI3K impairs differentiation and cytokine production, LY294002 enhances induction of IL-9 in Th9 cells. Importantly, Th9 cells exhibit substantial upregulation of FoxO1, and inhibiting FoxO1 in mice reverses the observed effects LY294002 has on IL-9 production (Malik and Awasthi 2018; Malik et al. 2017). Inhibition of PI3K/AKT induces expression of FoxO1, *Il9,* and other Th9-associated genes, even in un-polarized activated T cells (Malik and Awasthi 2018; Malik et al. 2017). Therefore, while FoxO1 is generally thought to antagonize effector cell differentiation, it appears to positively regulate Th9 differentiation in mice. In addition to DNA-binding domains, by which FoxO1 associates with the promoters of *Il9* and *Irf4* (Malik and Awasthi 2018), FoxOs also contain protein-interaction domains. In vitro protein-interaction assays have demonstrated the FoxO1-IRF4 protein complex to be important in the induction of Th9 transcription factors (Li et al. 2017). Pharmacological inhibition or genetic disruption of FoxO1 in CD4+ T cells results in a decrease in IL-9 expression both in vitro and in an allergic airway inflammation model (Buttrick et al. 2018).

mTOR mediates IL-9 induction (Wang et al. 2020), though whether this is dependent on input from PI3K requires further investigation. The mTORC1 target HIF-1α directly regulates IL-9 by binding the *Il9* promoter while mTORC1 itself promotes histone acetylation and IL-9 expression (Wang et al. 2020). In an OVA-induced mouse model of allergic airway inflammation, Th9 differentiation and IL-9 production were attenuated in Rictor-deficient T cells, and adoptive transfer of Rictor-deficient Th9 cells showed significantly reduced disease severity compared to transfer of wild-type Th9 cells (Chen et al. 2017). While findings that both mTOR and FoxO positively regulate Th9 differentiation may appear contradictory, studies have shown Rictor deficiency to impair Th9 differentiation by influencing IRF4 expression rather than affecting FoxO activity (Chen et al. 2017).

The cytokine IL-7 is important to T cell homeostasis, survival, and exerts a specific effect on Th9 differentiation via PI3K (Bi et al. 2017). Pre-treatment of murine CD4+ T cells with IL-7 prior to Th9 cell stimulation leads to a dose-dependent increase in IL-9-producing cells, while such effects are not observed upon pre-treatment with IL-2 or IL-15. Similar results were obtained in naïve human T cells, although gene

expression microarray analysis revealed little changes in Th9-associated transcription factors. Instead, IL-7 was shown to promote histone acetylation at the *IL9* locus in a PI3K/AKT/mTOR-dependent manner, resulting in enhanced gene transcription (Bi et al. 2017). However, the involvement of a specific PI3K isoform in this context was not evaluated.

Taken together, the role of individual PI3K isoforms in Th9 cells has not been well studied but PI3K signaling appears to play an important part in their generation (Fig. 2). Considering the implications of Th9 cells in human disease, understanding their differentiation and transcriptional regulation at the molecular level is vital. The AKT/FoxO axis is involved in the induction of IL-9, and deciphering the interplay between PI3K/AKT and SMAD-dependent signaling may provide critical insight as FoxO1 activity is also regulated in a TGF-β/SMAD-dependent manner (Buttrick et al. 2018).

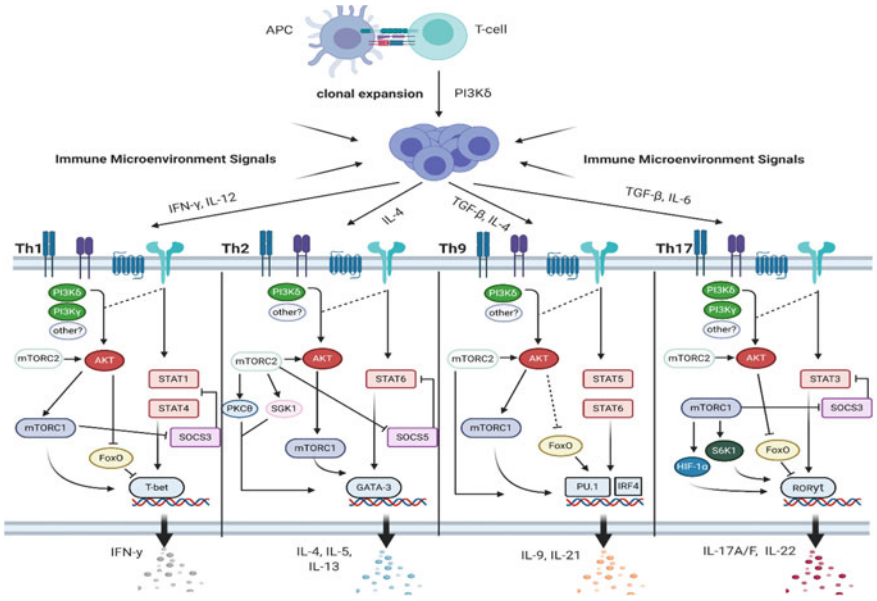

Fig. 2 PI3K control of CD4$^+$ T cell subset generation—created with BioRender.com. Naïve CD4$^+$ T cells are activated via interaction with APC. PI3Kδ controls clonal expansion and signals from the immune microenvironment shape T cell fate. IFN-γ and IL-12 generate Tbet expressing Th1 cells, while IL-4 generates Th2 cells which express GATA-3. TGF-β and IL-4 promote generation of Th9 cells in which PU.1 and IRF4 are among important transcription factors. Th17 cells express RORγt and are generated by TGF-β and IL-6. Downstream of cytokine receptors, JAK-STAT signaling promotes induction of lineage-specific gene-regulatory programs while SOCS antagonizes STAT proteins. Typically, IFN-γ and IL-4, IL-5, and IL-13 are produced by Th1 and Th2 cells, respectively. Th9 cells produce IL-9 and IL-21, and Th17 cells produce IL-17A/F and IL-22. The PI3K/AKT/mTOR pathway plays distinct roles in T cell subset generation, contributing to the induction of gene-regulatory programs, cytokine production, and lineage stability, with some but not all the proposed mechanisms depicted simplistically below

4.4 Th17 Cells

Th17 cells mediate immunity against extracellular pathogens. Their generation relies on IL-6, IL-21, IL-23, and TGF-β which generally promote STAT3-dependent induction of RAR-related orphan receptor gamma (RORγt), the Th17 master transcription factor (Saravia et al. 2019). Secretion of IL-17A/F, IL-21, and IL-22 by Th17 cells recruits neutrophils and macrophages to tissues, and Th17 cells are typically associated with inflammatory diseases including psoriasis, RA, MS, inflammatory bowel disease (IBD), and asthma (Bhaumik and Basu 2017; Tesmer et al. 2008; Wilke et al. 2011). Inhibitors of PI3Kδ and PI3Kγ attenuate Th17 responses in a number of animal models for diseases including psoriasis and RA (Stark et al. 2015; Vanhaesebroeck et al. 2016).

PI3Kδ plays a direct role in IL-17 production (Way et al. 2016). Inhibition of PI3Kδ in murine T cells stimulated in Th17 polarizing conditions results in a dose-dependent reduction in IL-17 production, with similar results obtained upon inhibition of AKT (Way et al. 2016). Therefore, IL-17 production in TCR/CD28-stimulated T cells relies on PI3Kδ-mediated activation of AKT. Notably, FoxO1 negatively regulates Th17 differentiation by inhibiting RORγt via its DNA-binding domain and antagonizes *Il17a* transcription (Laine et al. 2015), and FoxO1-deficient mice show increased production of IL-17A (Kerdiles et al. 2009). Therefore, the PI3Kδ/AKT/FoxO axis is crucial in early induction of the Th17 program. Th17 cells also show a selective requirement for mTORC1 and deletion of *Rheb*, an upstream activator of mTORC1, or *Raptor*, an essential subunit of mTORC1, strongly impairs Th17 cell differentiation in mice (Kurebayashi et al. 2012; Nagai et al. 2013). HIF-1α, a downstream target of PI3K/AKT enhances *Il17* transcription via physical association with RORγt (Capone and Volpe 2020) and also induces aerobic glycolysis, which Th17 cells are particularly reliant on for their pro-inflammatory phenotype (Dang et al. 2011). Another target of PI3K/AKT, ribosomal protein S6 kinase beta-1 (S6K1), positively regulates Th17 differentiation by promoting early growth response protein-2 EGR2-mediated suppression of *Gfi1*, a zinc-finger domain protein that activates RORγt (Kurebayashi et al. 2012). At the same time, PI3Kδ-derived signals increase levels of S6K2, the nuclear counterpart of S6K1, which enhances nuclear translocation and activity of RORγt (Kurebayashi et al. 2012). Further, mTORC1 inhibits SOCS3 and enhances STAT3-dependent gene transcription, emphasizing again the crosstalk between mTOR complexes and STAT/SOCS networks (Saleiro and Platanias 2015; Nagai et al. 2013).

Given the central role of Th17 cells in inflammatory disorders, studies targeting PI3Kδ and or PI3Kγ have shown promising results. IL-17 is strongly associated with psoriasis (Tesmer et al. 2008; Brembilla et al. 2018), and both $p110\delta^{D910A/D910A}$ mice and PI3Kγ knockout mice show protection in an imiquimod-induced psoriasis-like dermatitis model, which correlates with lower levels of IL-17 in the lesions and serum and decreased IL-17-producing cells (Roller et al. 2012). Further, inhibition of PI3Kγ but not PI3Kδ blocked CCL20-dependent chemotaxis of CCR6+IL-17A-producing

T cells in mice and humans, which is important in recruiting inflammatory cells to psoriatic lesions (Roller et al. 2012).

While PI3Kγ appears to be dispensable for Th17 cell differentiation, mice deficient in PI3Kγ showed reduced T cell priming and trafficking to peripheral inflammatory sites and exhibited delayed onset of EAE (Berod et al. 2011). On the other hand, $p110\delta^{D910A/D910A}$ mice show reduced Th17-responses, decreased IFNγ and IL-17A production, and exhibit greater effector cell apoptosis (Haylock-Jacobs et al. 2011). In contrast to $PI3K\gamma^{-/-}$ mice which show impaired dendritic cell (DC) migration and activation of adaptive immunity (Prete et al. 2004), the observations in $p110\delta^{D910A}$ mice were shown to be independent of DC function and instead due to Th17 cell-intrinsic defects. Animal models of arthritis have shown selective PI3Kδ or PI3Kγ and also dual inhibitors to alleviate clinical signs of disease (Camps et al. 2005; Winkler et al. 2013). Further, therapeutic administration of the dual p110α/δ inhibitor ETP-46321 inhibited responses to collagen-induced arthritis, reduced IL-17 production, and alleviated clinical symptoms in vivo (Aragoneses-Fenoll et al. 2016). Collectively, dual inhibition of PI3Kδ and PI3Kγ seems to attenuate inflammation more potently by targeting both Th17 cell activation and chemotactic responses.

In summary, PI3K signaling is important in induction of the Th17 gene-regulatory program and is also directly involved in Th17-related cytokine production. Th17 cells are widely implicated in the pathogenesis of chronic inflammatory disorders and selective inhibitors of PI3K have shown efficacy in several Th17-mediated preclinical models. Uncovering how PI3Kδ and PI3Kγ coordinate pathogenic inflammatory processes in Th17 cells and the potential role of other PI3K subunits in Th17 generation and function will be critical in the development of new therapies (Fig. 2).

5 Conclusion

This chapter aims to reveal the diverse functions of PI3K signaling in Th subsets and justify current efforts to continue elucidating the role and regulation of PI3K isoforms in T cell-dependent processes. Study of Tregs and Teffs has shown that the PI3K/AKT pathway is a critical signaling hub that determines the balance between immune tolerance and inflammation (Pompura and Dominguez-Villar 2018), implying that differential PI3K signaling may be exploited therapeutically. The PI3K pathway plays an essential role in nearly all aspects of lymphocyte biology, and the restricted expression of PI3Kδ and PI3Kγ isozymes in these cells has sparked the development of a number of selective and dual PI3K inhibitors to alleviate autoimmune disease, inflammatory disorders, and cancer. The robustness of our immune system requires T helper responses to be highly specific and tightly controlled in a spatial and temporal manner to avoid immunopathology and maintain homeostasis. T cell-intrinsic control of cellular differentiation and function relies on crosstalk between intracellular signaling pathways, and dynamic regulation of transcriptional and translational networks which reflects the integration of diverse extracellular cues. The

seemingly promiscuous stimuli that activate PI3Ks, the broad range of PH domain-containing effectors, and the cell-specific regulation of PI3K-signaling are some of the issues that have precluded an in-depth characterization of PI3K isoforms in different T cell subsets. Elucidating how distinct and synchronous signals are transduced, particularly by PI3Kδ and PI3Kγ, will provide crucial insight into the pathogenic processes which arise from the integration of these signaling pathways.

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PI3K Isoforms in CD8$^+$ T Cell Development and Function

Pankaj Gaur, Mikayel Mkrtichyan, Vivek Verma, Nazli Jafarzadeh, Mariana Hattar, Seema Gupta, and Samir N. Khleif

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Abstract CD8$^+$ T cells are an essential part of the immune system and play a vital role in defending against tumors and infections. The phosphoinositide-3-kinase (PI3K), especially class I, is involved in numerous interrelated signaling pathways which control CD8$^+$ T cell development, maturation, migration, activation, and differentiation. While CD8$^+$ T lymphocytes express all class I PI3K isoforms (PI3Kα,

P. Gaur · M. Mkrtichyan · V. Verma · N. Jafarzadeh · M. Hattar · S. Gupta · S. N. Khleif (✉)
The Loop Immuno-Oncology Laboratory, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, USA
e-mail: snk48@georgetown.edu

P. Gaur
e-mail: pg684@georgetown.edu

M. Mkrtichyan
e-mail: mm4530@georgetown.edu

V. Verma
e-mail: vv149@georgetown.edu

N. Jafarzadeh
e-mail: nj387@georgetown.edu

M. Hattar
e-mail: mnh47@georgetown.edu

S. Gupta
e-mail: sg1335@georgetown.edu

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PI3Kβ, PI3Kδ, and PI3Kγ), isoform-specific functions, especially for PI3Kα and PI3Kβ have not been fully elucidated. A few studies suggest the important role of p110δ and p110γ in $CD8^+$ T cell activation, signaling, chemotaxis and function and several clinical trials are currently testing the effect of isoform-specific inhibitors in various types of cancers, including Indolent Non-Hodgkin Lymphoma, Peripheral T cell Lymphoma, Chronic Lymphocytic Leukemia, Small Lymphocytic Lymphoma, non-small cell lung carcinoma (NSCLC), head & neck cancer, and breast cancer. This chapter summarizes current knowledge of the roles of various PI3K isoforms and downstream signaling pathways in regulating $CD8^+$ T cell fate, including cell proliferation, migration, and memory generation. We also discuss certain clinical trials employing PI3K inhibitors for cancer therapy, their limitations, and future perspectives.

The phosphoinositide-3-kinase (PI3K) family plays a major role in nearly all aspects of cellular biology and is a key node in lymphocyte behavior and immune response (Dituri et al. 2011). PI3K isoforms have been divided into three classes (class I, class II and class III) based on their structural characteristics and preferred lipid substrate. Among the PI3K isoforms, class I PI3Ks (PI3Kα, PI3Kβ, PI3Kδ, and PI3Kγ) play an important role in lymphocyte biology through T cell receptor (TCR) signaling, especially in T cell survival, development, proliferation, and differentiation (Kim and Suresh 2013; Okkenhaug et al. 2014). $CD8^+$ T cells are a subset of T lymphocytes with a specific capacity to selectively target and kill cells infected with intracellular pathogens as well as tumor cells. $CD8^+$ T cells play a critical role in defending against cancers via antigen-specific tumor cell killing. The development and maturation of antigen-specific $CD8^+$ T cells are complex processes involving numerous interrelated signaling pathways (Kim and Suresh 2013; Murter and Kane 2020). In $CD8^+$ T cell activation, engagement of the TCR with MHC class I/antigen complex along with costimulatory receptors triggers signaling pathways that lead to activation, expansion, and differentiation. This chapter will focus on the critical effects of PI3K isoforms on $CD8^+$ T cell proliferation, survival, migration, and effector memory subset formation.

1 Activation of PI3K in $CD8^+$ T Cells

In T cells, the best defined receptor for PI3K recruitment is the costimulatory protein CD28, which is a transmembrane protein that activates naïve T cells via interaction with B7 family proteins expressed by antigen-presenting cells (APCs) (Garçon et al. 2008; Boomer and Green 2010). The cytoplasmic regions of CD28 bind p85 subunits, and the ligation of both antigen receptors and costimulatory receptors strongly activates PI3Ks. Adaptor proteins such as linkers for activation of T cells (LAT) mediate tyrosine phosphorylation and activate class IA PI3Ks downstream of antigen and

costimulatory receptors. Toll-like receptors (TLRs), interleukin (IL)-1 receptors, and members of the tumor necrosis factor receptor (TNFR) family (including CD40), also activate class IA PI3Ks in macrophages and dendritic cells (DCs) (Koyasu 2003). However, the molecular signaling mediated by these receptors, leading to activation of downstream PI3Ks in CD8+ T cells is not well elucidated. Different cytokines such as IL-2, IL-3, IL-6, IL-7, IL-15, granulocyte colony-stimulating factor, and interferons (IFNs) also play an important role in activating the class I PI3Ks in T cells (Koyasu 2003; Gagnon et al. 2008).

Activation of the catalytic subunits downstream of PI3Ks leads to the catalytic conversion of phosphoinositide (PI)-(4,5)-P2 to the membrane-bound second messenger PI-(3,4,5)-triphosphate (PIP3). PIP3 recruits several pleckstrin homology (PH) proteins such as the protein kinase B family (PKB aka AKT family, there are three AKT family members in mammals, AKT1–3) and phosphoinositide-dependent kinase 1 (PDK1) (Manning and Toker 2017), considered to be a chief downstream target of PI3K. AKT activation results in the phosphorylation and activation of a multitude of downstream pathways (Hers et al. 2011; Basu and Lambring 2021). AKT phosphorylation leads to mTORC1 activation, which plays a role in protein synthesis (Delgoffe et al. 2011), cell growth, and metabolism (Laplante and Sabatini 2012). AKT also directly phosphorylates glycogen synthase kinase 3 (GSK3), leading to its inactivation and therefore, a decrease in cellular glycogen synthesis. GSK3 also regulates cell survival in CD8+ T cells by phosphorylating cyclins and transcription factors c-jun and c-myc (Kim and Suresh 2013).

PI3K through AKT also phosphorylates and inactivates forkhead box O (FOXO) transcription factors in the nucleus, thus inhibiting the expression of FOXO target genes involved in proliferation, apoptosis, motility, and metabolism (Hedrick et al. 2012). The PI3K pathway also interfaces with other signaling pathways such as the canonical Wnt/β-catenin, NFκB (nuclear factor κ B), and the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways (Manning and Cantley 2007; Delgoffe et al. 2011). Increased PIP3 levels lead to a biphasic elevation in the cytosolic calcium concentrations and increased translocation and activation of NFAT (nuclear factor of activated T cells) transcription factors. NFAT binds to the promoters of most cytokine genes and promotes cytokine production, thus affecting the activation of T cells (Fig. 1).

2 Role of PI3K Pathway in CD8+ T Cells

PI3K and its downstream effectors coordinate the convergence of the CD8+ T cell fate-determining pathways and diverse cellular processes. There are several reports that investigated the role of PI3K as well as those of its downstream effectors, AKT, mTOR, FOXOs, and GSK3 in CD8+ T cell homeostasis (Araki et al. 2009; Kerdiles et al. 2009; Rao et al. 2010; Kim and Suresh 2013; Murter and Kane 2020).

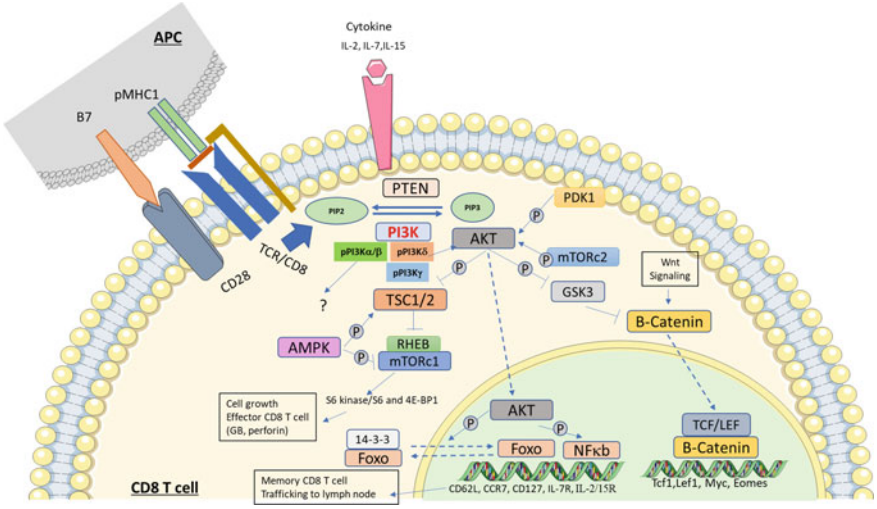

Fig. 1 Activation and function of PI3K signaling in CD8$^+$ T cells. TCR engagement by peptide/MHC I complex activates PI3K in CD8$^+$ T cells. Signals from stimulation, cytokines, and chemokines can also activate PI3K. PI3K phosphorylates PIP2 to generate PIP3, which recruits AKT and PDK1. Full activation of AKT requires phosphorylation by both PDK1 and mTORC2. In the cytosol, AKT phosphorylates and inhibits the negative regulatory TSC1/2 complex of mTORC1, which promotes mTORC1-mediated protein synthesis and cell growth through modulating S6K and 4E-BP. Cytosolic AKT also inhibits GSK3 and regulates the expression of Wnt/β-catenin pathway targets lef1, myc and Eomes. Besides, AKT translocates to the nucleus, phosphorylates FOXO, leading to its translocation to the cytosol from the nucleus. This results in the transcriptional activation of multiple transcription factors that are important for proliferation, metabolism, motility, and apoptosis. AKT phosphorylation also activates IKK and NFκB-dependent transcription and controls CD62L, CCR2, CD127 expression and differentiation into memory CD8$^+$ T cells. Effects of PI3K signaling via the PI3K isoforms PI3Kα and PI3Kβ require further exploration

2.1 CD8$^+$ T Cell Migration

The peripheral trafficking of activated T cells is mediated by adhesion molecules and chemokine receptors, including CD62L, CXCR3, CCR3-7, CCL19, and CCL21 (Nolz et al. 2011). The p110γ catalytic subunit of PI3K plays a significant role in chemokine responses and promotes PIP3 signaling. T cells from p110γ-deficient mice show reduced chemotactic responses to the lymphoid chemokines, CCL19, CCL21, and CXCL12 (Martin et al. 2008). Martin et al. (2008) reported that the migration and trafficking of naive CD8$^+$ T cells into secondary lymphoid organs in vivo was unaffected by the loss of p110γ. Additionally, loss of p110γ did not affect CD8$^+$ T cell proliferation and effector cell differentiation in vitro and in vivo. However, the p110γ isoform is required for the chemotaxis and migration of effector CD8$^+$ T cells to infection sites (Martin et al. 2008; Vivier et al. 2011). It is essential to mention that factors that influence T cell migration and/or homing, the recruitment of primed T cells to non-lymphoid tissues, and their retention at the site

are enhanced by various inflammatory signals, in addition to TCR engagement by antigen-displaying endothelium and resident APCs. Mouse T lymphocytes with inactive p110δ showed normal constitutive trafficking and migratory responses to nonspecific stimuli. However, these cells lost susceptibility to TCR-induced migration and failed to localize efficiently to target tissue (Jarmin et al. 2008). Jarmin et al. (2008) showed that antigen-induced T cell trafficking and subsequent inflammation were retracted by inhibition of PI3K p110δ activity. Thus, PI3Kγ controls T cell migration in response to chemokines, and PI3Kδ regulates the expression of adhesion and chemokine receptors on activated T cells (Kim and Suresh 2013). Macintyre et al. (2011) demonstrated that high levels of AKT activation down-regulate the expression of adhesion molecules, CD62L, CCR7, and sphingosine-1-phosphate receptor (S1PR), thus redirecting the trafficking of effector CD8+ T cells away from the lymph nodes and to the site of inflammation (Macintyre et al. 2011). Inhibition or genetic depletion of PDK1 prevents the downregulation of FOXO target genes encoding proteins essential for naïve CD8+ T cell trafficking: IL-7 receptor α subunit (CD127), L-selectin (CD62L), transcription factor KLF2, CCR7, and S1PR1 (Sinclair et al. 2008; Macintyre et al. 2011).

2.2 *Role of PI3K in CD8+ T Cell Development, Survival, and Proliferation*

For the acquisition of a complete mature peripheral T cell phenotype, T cell progenitors undergo a series of tightly regulated developmental events in the thymus that depend on the integration of signaling cascades downstream of the TCR. However, it remains unclear which signaling pathway is most critical for survival during the transition from the CD4/CD8 double-negative (DN) to double-positive (DP) stage. PI3K is one of the most common pathways to all receptor signal transduction cascades. In particular, PI3Kδ and PI3Kγ isoforms are known to play critical roles during T cell development (Hawkins and Stephens 2015). Studies show that knockdown of PI3Kδ or PI3Kγ in mice leads to impaired proliferation during T cell development, but deletion or inactivation of both isoforms during thymopoiesis results in a block at the DP cell stage (Webb et al. 2005; Swat et al. 2006; Finlay 2012; Okkenhaug 2013). PI3Kδ is not required for CD8+ T cell proliferation or associated metabolic changes such as increased glucose uptake during their maturation. However, PI3Kδ can regulate the homeostatic trafficking of CD8+ T cells to the lymph nodes and contribute to the acquisition of full effector functions and migration to peripheral tissues (Lucas et al. 2016). When CD8+ T cells differentiate from naïve to effector cells, they lose their ability to produce IL-2 (Sallusto et al. 1999). Abu Eid et al. (2017) showed that PI3Kδ inhibition in vitro using CAL-101 maintains a high level of IL-2 secretion and CD62L expression in mouse and human CD8+ T cells (Abu Eid et al. 2017). Furthermore, they demonstrated that the expansion of antigen-primed CD8+ T cells cultured in the presence of IL-2 is not affected by the p110δ inhibitor, which is in agreement

with another study from Sinclair and colleagues (Sinclair et al. 2008). These studies revealed a selective role for p110δ in IL-2 signal transduction in that it is required for IL-2-mediated downregulation of CD62L but not for IL-2-induced expression of nutrient receptors, mitogenesis, or cell growth. PI3Kδ inhibition improves the proliferative ability and survival of $CD8^+$ T cells by preserving the memory phenotype (Sinclair et al. 2008; Abu Eid et al. 2017). Studies have shown that an overactive p110δ PI3K in $CD8^+$ T cells leads to their rapid proliferation and differentiation, resulting in chronic inflammation and a greater risk of viral infection. However, this pathway had an important role in the formation of long-term memory $CD8^+$ T cells. This was proven by using an inactive mutant of p110δ subunit in mice which led to the poor proliferation. Altogether, data suggest that PI3Kδ plays a vital role in $CD8^+$ T cell fate and function (Liu et al. 2009; Gracias et al. 2016; Abu Eid et al. 2017).

2.3 $CD8^+$ T Cell Activation, Differentiation, and Memory

$CD8^+$ T cells are a crucial component of the adaptive immune response, regulating immunity to intracellular pathogens and tumors. During viral or intracellular bacterial infections, antigen-specific $CD8^+$ T cells are activated, which then proliferate rapidly into differentiated effector $CD8^+$ T cells. The role of specific PI3K isoforms in the differentiation of $CD8^+$ T cells is still not well known. There are few reports that tested whether inhibition of a single PI3K isoform would be sufficient to delay terminal differentiation of $CD8^+$ T cells (Gracias et al. 2016; Abu Eid et al. 2017). Pharmacological inhibition of PI3Kα (A66) or PI3Kβ (TGX-221) showed no differences in the phenotype of pMel-1 $CD8^+$ T cells, but inhibition of PI3Kδ (CAL-101) displayed a higher percentage of central memory T cells (T_{CM}), a similar phenotype observed with the pan-PI3K inhibitor GDC-0941 (Abu Eid et al. 2017) (Table 1). PI3K signaling is required in activated $CD8^+$ T cells to initiate the transcriptional program for cytotoxic $CD8^+$ effector functions via regulation of cytotoxic effector molecules, including granzymes and perforin expression, and production of proinflammatory cytokines (Abu Eid et al. 2017). However, the role of PI3K signaling in controlling the cytotoxic capacity of mature effector $CD8^+$ T cells has not been studied in detail. Abu Eid and colleagues showed that PI3Kα, β, or δ isoform-specific inhibition (using A66, TGX-221, and CAL-101, respectively) in vitro did not affect the ability of mouse $CD8^+$ T cells to produce TNF, IFNγ, and granzyme B, though PI3Kδ inhibition could delay terminal differentiation of $CD8^+$ T cells and maintain the memory phenotype (Abu Eid et al. 2017). They also demonstrated that ex vivo PI3Kδ inhibition enhances antitumor therapeutic ability of adoptively transferred $CD8^+$ T cells in animal models. A recent study showed that in a syngeneic A20 lymphoma model, PI3Kδ inhibition by parsaclisib increased the percentage of effector memory ($CD44^{high}CD62L^{low}$) but decreased the percentage of central memory ($CD44^{high}CD62L^{high}$) $CD4^+$ and $CD8^+$ T cells in the spleen, in contrast to the role of PI3Kδ in the regulation of T cell differentiation described by Abu Eid et al. (2017). This discrepancy could be due to multiple reasons, including different

Table 1 IC_{50} of different reported p110 isoforms inhibitors

	p110α	p110β	p110δ	p110ϒ
A66	32 nM	–	>1.25 μM	
TGX-221	–	5 nM	0.1 μM	–
Idelalisib (CAL-101, GS-1101)			2.5 nM	89 nM
IC87114	>50 mM	>50 mM	500 nM	>20 nM
AS252424	935 nM	>10 mM	>10 mM	30 nM
BKM120	52 nM	166 nM	116 nM	262 nM
Pictilisib (GDC-0941, RG7321)	3 nM	3 nM	33 nM	75 nM
Taselisib (GDC 0032, RG7604)	0.29 nM	9.1 nM	0.12 nM	0.97 nM
Ly294002	500 nM	973 nM	570 nM	
Voxtalisib (SAR245409, XL765)	39 nM	43 nM	113 nM	3 nM
PI-103	2 nM	3 nM	3 nM	15 nM
Eganelisib (IPI-549)				16 nM
Duvelisib			1 nM	50 nM

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experimental systems carried out for the assessment of memory generation after PI3K inhibition (ex vivo (Abu Eid et al. 2017) and in vivo (Shin et al. 2020)), and different PI3Kδ inhibitors and doses. However, Shin, Stubbs et al. also noted that the percentage of tumor-infiltrating central and effector memory CD8$^+$ T cells was not affected, which could be controlled by the effect of the complex cellular interactions on PI3K signaling in the tumor microenvironment (Shin et al. 2020). Recently, another study showed that dual PI3Kγ and PI3Kδ blockade impairs CD8$^+$ T cell function as shown by the reduced antitumor activity of adoptively transferred CD8$^+$ T cells that were treated with dual PI3K inhibitors in B16F10 tumor-bearing mice (Dwyer et al. 2020). Both inhibitors impacted T cell cytokine production after primary and secondary stimulation with tumor antigen, and dual isoform-inhibited (IPI-145) pmel-1T cells were the most compromised in their capacity to secrete granzyme B, IL-2, and TNF, even after cells were reactivated with antigen. Carnevalli et al. reported that PI3Kα/δ inhibitors (AZD8835) enhanced CD8$^+$ T cell activation as shown by elevated expression of the activation marker CD69 and increased IL-2 production without limiting their proliferation (Carnevalli et al. 2018). As activated T cells produce autocrine/paracrine IL-2 as part of a feed-forward loop to reinforce their efficient activation, this study showed that PI3Kα/δ inhibitors (AZD8835) directly enhance effector CD8$^+$ T cell activity through increased production of IL-2 and in turn enhanced survival of AZD8835-treated T cells.

The PI3K/AKT axis plays an integral role in memory formation. AKT phosphorylates and sequesters FOXO transcription factors preventing the transcription of *CD62L*, CCR7, *CD127*, and other molecules associated with memory T cells (Hedrick et al. 2012). Kim et al. demonstrated that sustained AKT activation initiates a transcriptional program that regulates terminal differentiation of CD8$^+$ T cells

and reduces $CD8^+$ T cell memory due to less activity of FOXO and downregulation of the Wnt/β-catenin pathway (Kim et al. 2012). In line with these observations, inhibition of AKT increases the number of memory $CD8^+$ T cells (Abu Eid et al. 2015). Interestingly, inhibition of downstream mTOR augments the functional quality of cytotoxic $CD8^+$ T cell responses by prompting a $CD8^+$ T cell memory phenotype including the stem cell-like memory (Li et al. 2012; Scholz et al. 2016; Borsa et al. 2019; Verma et al. 2021). Treatment with the pan-PI3K inhibitor GDC-094 also increased the frequency of central memory T cells (T_{CM}) cells in pMel-1 $CD8^+$ T cells (Abu Eid et al. 2017). However, only PI3Kδ inhibition shows a similar phenotype observed with pan-PI3K inhibition, suggesting that PI3Kδ is the only isoform responsible for the terminal differentiation of $CD8^+$ T cells. However, mechanisms that enhance the memory phenotype and delay the terminal differentiation of $CD8^+$ T cells without significantly impacting their proliferation and function still warrant further investigations (Table 2).

Table 2 Summary of the roles of PI3Ks and possible exploitation of isoform-selective PI3K functions in a therapeutic context

Class IA PI3K	Function in $CD8^+$ T cells	References
p110α inhibition	– Impairs hematopoietic stem cell activation – $CD8^+$ T effector profiles	Carnevalli et al. (2018), Hemmati et al. (2019)
p110β inhibition	– No effect on T cells	Abu Eid et al. (2017)
p110δ inhibition	– Expression of adhesion and chemokine receptors expressed in activated T cells – TCR-induced migration – DN stage of T cell development – $CD8^+$ T cell differentiation – Reduced IL-2 secretion – Proliferation and survival – Treg/$CD8^+$ T cell ratio – Central memory generation – Restoration of the CAR-T effector/memory ratio	Jarmin et al. (2008), Macintyre et al. (2011), Finlay (2012), Gracias et al. (2016), Abu Eid et al. (2017), Aragoneses-Fenoll et al. (2018), Carnevalli et al. (2018), Zheng et al. (2018), Munoz et al. (2021)
p110γ inhibition	– T cell development – Chemotaxis and migration of effector $CD8^+$ T cells – Impaired TCR/CD28-mediated activation	Martin et al. (2008), Vivier et al. (2011), Finlay (2012), Ladygina et al. (2013)

2.4 *Effect of PI3K Downstream Signaling on the Regulation of CD8$^+$ T Cell Memory*

The signaling pathways that regulate the development of memory T cell subsets are not fully understood. Still, understanding these pathways is essential for the development of more productive CD8$^+$ T cells against tumors and intracellular pathogens. AKT (AKT1, AKT2, AKT3) is the crucial downstream effector of PI3K signaling (Basu and Lambring 2021). AKT can translocate from the cell membrane to the cytosol and nucleus, where it controls a large number of critical signaling pathways (Manning and Toker 2017). AKT1 and 2 isoforms have been shown to drive the terminal differentiation of antigen-specific CD8$^+$ T cells. The inhibition of AKT1 and 2 isoforms via AKT1/2 inhibitors in CD8$^+$ T cells delays the exhaustion of CD8$^+$ T cells, prolongs their survival, preserves and maintains a high expression of CD62L and CD127 (memory phenotype), and significantly increases their proliferative potential after antigenic rechallenge (Abu Eid et al. 2015). A recent study used different PI3Kγ (IPI-549), PI3Kδ (CAL-101 or TGR-1202), or dual PI3Kγ/PI3Kδ isoform-specific (IPI-145) inhibitors to measure phosphorylation of AKT at Ser 473 and Thr 308 in pMel-1 CD8$^+$ T cells over time as a measurement of AKT activation in vitro. All inhibitors reduced the phosphorylation of AKT at Ser 473 and Thr 308, with pAKT Thr 308 remaining low even 10 min after addition of the inhibitor (Dwyer et al. 2020). AKT phosphorylation of serine and/or threonine amino acids modulates the expression and function of multiple downstream signaling molecules. Two negative regulators of AKT, tuberous sclerosis complex 2 (TSC2) and proline-rich AKT substrate 40 kDa (PRAS40) activate mTORC1, which controls protein synthesis, cell growth, and metabolism (Laplante and Sabatini 2012). PI3K, through AKT, inactivates TSC1/TSC2 and stimulates the small Ras-related GTPase Rheb, which directly triggers mTORC1 activity and promotes protein translation through 4E-BP and S6K (Laplante and Sabatini 2012). Several studies have shown that mTORC1 might limit the differentiation of memory CD8$^+$ T cells (Araki et al. 2009; Rao et al. 2010; Restifo et al. 2012; Sowell and Marzo 2015). In antigen-stimulated naïve CD8$^+$ (OT-I) T cells, mTORC1 negatively regulates the differentiation of memory precursor effector cells (MPECs) and differentiation into memory CD8$^+$ T cells (Rao et al. 2010). Inhibition of mTORC1 via rapamycin enhanced the development of MPECs and improved the number of memory CD8$^+$ T cells, even at the time of infection (Araki et al. 2009). mTORC1 activity also promotes the terminal differentiation of effector CD8$^+$ T cells by increasing the Tbet: Eomes ratio (Rao et al. 2010). Dwyer and colleagues also evaluated other signaling events induced by TCR activation, such as MAPK, mTOR, and STAT5 activation (Dwyer et al. 2020) and found that the inhibition of PI3Kγ, PI3Kδ, or both PI3Kγ and PI3Kδ transiently blocked components of the PI3K signaling axis in T cells but did not mediate off-target effects on other pathways including mTOR or STAT5.

As described above, PI3K/AKT signaling inactivates GSK3 via its phosphorylation, which increases the activity of transcription factors c-jun and c-myc (Hers et al. 2011). Moreover, AKT phosphorylates and inactivates GSK3β, which results

in stabilization and nuclear localization of cytosolic β-catenin. GSK3β thus provides crosstalk between Wnt signaling and the PI3K signaling pathways (Kim and Suresh 2013; Dimou and Syrigos 2020). Moreover, data suggest that GSK3β inhibition leads to Wnt signaling-mediated reduction of effector cell's terminal differentiation and promotion of memory $CD8^+$ T cell development (Gattinoni et al. 2009). As GSK3 regulates c-myc, studies have shown that lef1 and myc expression are highest in naïve and central memory $CD8^+$ T cells but are substantially downregulated in terminally differentiated effector cells (Hers et al. 2011; Kim et al. 2012). Costimulatory signals (CD28 and OX40) regulate c-myc that in turn modulates the generation of $CD8^+$ memory T cells during viral infection (Haque et al. 2016). Inhibition of c-myc by BET bromodomain inhibitors promoted expansion of both naïve and $CD45RO^+$ memory T cell phenotypes, and adoptive transfer of inhibitor-treated $CD8^+$ T cells showed extended survival in a T-ALL mouse model (Kagoya et al. 2016), further confirming the role of c-myc in memory generation. Besides controlling the activities of mTORC1 and GSK3, AKT also phosphorylates and inactivates FOXO transcription factors, which stimulates the transcription of the memory markers *CD127* and *CD62L*, memory transcription factor Eomes and inhibits the effector transcription factor Tbet in $CD8^+$ T cell (Rao et al. 2012; Michelini et al. 2013; Delpoux et al. 2017).

The PI3K/AKT pathway indirectly controls cellular functions by interacting with other signaling pathways such as the NFκB and JAK/STAT pathways (Okkenhaug and Vanhaesebroeck 2003; Kim and Suresh 2013). PI3K enhances the transactivation of NFκB through PDK1, a downstream kinase of PI3K that phosphorylates IKKβ and activates NFκB signaling. Inhibition of p110α/γ PI3K results in a dramatic suppression of downstream signaling events, including AKT phosphorylation, IKK activation, and NFκB-mediated transcription (Dagia et al. 2010). Loss of AKT during T cell activation reduces NFκB binding to its target gene promoters and diminishes the expression of TNF and IL-6 (Cheng et al. 2011). Failure in maintaining NFκB signals leads to impaired Eomes expression and a defect in $CD8^+$ T cell memory maintenance. Knudson et al. showed that the NFκB/Pim-1/Eomesodermin axis is required to maintain memory $CD8^+$ T cell longevity and effector function. Therefore, PI3K/AKT may further regulate the differentiation of $CD8^+$ T cells through the NFκB pathway (Knudson et al. 2017).

Cytokines such as IL-7 and IL-15 trigger the JAK/STAT and PI3K/AKT signaling pathways, which in turn modulate the differentiation of memory $CD8^+$ T cells (Schluns and Lefrançois 2003; Hand et al. 2010). A recent study showed that $CD8^+$ T cells display higher levels of phosphorylated AKT in Ser 473 and Thr 308 residues in response to IL-15 (Watkinson et al. 2021). IL-15 through JAK3 and PI3K signaling pathways can also activate telomerase reverse transcriptase (TERT) expression in memory $CD8^+$ T cells (Li et al. 2005; Barsov 2011; Watkinson et al. 2021). Cui et al. have demonstrated that in response to IL-15, long-lived memory precursor cells show significantly higher activation of AKT than short-lived effector cells. AKT phosphorylation induced by IL-2, IL-7, and IL-15 could be inhibited by the PI3K pan-inhibitor LY294002, indicating that cytokine-induced AKT phosphorylation was PI3K-dependent. Conversely, constitutive STAT5 activation dramatically

enhanced effector CD8$^+$ T cell survival and memory CD8$^+$ T cell formation after infection (Hand et al. 2010). Together, these data suggest that complex cytokine milieu can modulate PI3K/AKT signaling affecting the differentiation and memory generation of CD8$^+$ T cells.

3 PI3K Pathway and Lessons from Clinical Trials

PI3K isoforms are one of the key therapeutic targets in cancer immunotherapy based on the observations that inhibition of PI3K results in the regression of tumor progression in different mouse models, a decrease in the suppressive tumor microenvironment (with a direct effect on Tregs), an increase in the frequency of tumor-infiltrating effectors cells, and an enhanced memory potential of CD8$^+$ T cells (Stark et al. 2015; Okkenhaug et al. 2016; Fruman et al. 2017; Carnevalli et al. 2018; Murter and Kane 2020). Among all PI3K inhibitors, Idelalisib, Alpelisib, and Duvelisib have been approved by the Food and Drug Administration (FDA) to treat cancers (Meng et al. 2021). Several ongoing or completed clinical trials with PI3K-targeted therapies have shown promising therapeutic outcomes as summarized in Table 3. However, a recent clinical trial using PI3Kδ inhibitor, Idelalisib (GS-1101; CAL-101, NCT01090414), has been put on hold due to a significant incidence of infections owing mainly to the substantial risk of systemic toxicity (Murter and Kane 2020). Since the later stages of CD8$^+$ T cell differentiation in response to specific antigens are PI3Kδ-dependent, the use of PI3Kδ inhibitors may reduce the antiviral response due to CD8$^+$ T cell deprivation (Abu Eid et al. 2017). These results are consistent with other studies showing that either knockout/inactivation of the PI3K catalytic subunit p110δ or the PI3Kδ-selective inhibitor IC87114, impair CD8$^+$ T cell responses to both bacterial and viral infections (Pearce et al. 2015; Gracias et al. 2016). Other clinical trials are now focused on combining isoform inhibitors or utilizing pan or dual inhibition of PI3K/mTOR signaling, for example, Duvelisib (IPI-145) and Copanlisib (BAY 80–6946).

Duvelisib (IPI-145, NCT01476657), an inhibitor of PI3Kδ/γ isoforms, is currently in clinical development. PI3K-δ/γ inhibition may directly inhibit malignant T cell growth, making Duvelisib a promising candidate for patients with peripheral (PTCL) or cutaneous (CTCL) T cell lymphoma (Horwitz et al. 2018). Previous studies have shown that PI3K p110δ and p110γ are essential for cytokine production by immune effector cells (Carnevalli et al. 2018). Although Duvelisib did not cause cytotoxicity to T cells, it inhibited TNF and IFNγ production, cytokines needed for survival of CLL (chronic lymphocytic leukemia) cells. However, TNF and IFNγ are also crucial for the normal inflammatory response, and the inhibitory effect could be associated with opportunistic infections (Dong et al. 2014). In a preclinical model of T cell lymphoma (phase I trial), the selective inhibition of PI3Kγ reduced the immune-suppressive function of myeloid cells and promoted the transition of M2 macrophages (tumor-promoting) to an M1 phenotype (immune-stimulatory) in the TME. This

Table 3 Clinical trials targeting PI3K

Compound	Phase I	Phase II	Phase III	With FDA (on hold)	FDA approved
Isoform-specific PI3K inhibitor	AZD8186 (AstraZeneca) β/δ KA2237 (Karus Therapeutics) β/δ GS-9820/CAL-120 (Gilead) β/δ ME401/PWT-143 (MEI Pharma) δ	AMG 319 (Amgen) δ GSK2636771 (GlaxoSmithKline) β INCB050465/Parsaclisib (Incyte) δ Serabelisib/INK-1117 (Takeda) α Umbralisib/TGR-1202 (TG Therapeutics) δ RP6530/Tenalisib(Rhizen Pharmaceuticals) δ/γ	GDC-0032/Taselisib (Genentech) α/δ/γ BYL719/Alpelisib (Novartis) α	CAL-101/idelalisib (Gilead) δ	Duvelisib/IPI-145 (Infinity) δ/γ
Pan-PI3K inhibitor	CH5132799 (TohokuNiproPharm)	XL147/ Pilaralisib (Sanofi) ZSTK474 (Zenyaku Kogyo)	BKM-120/Buparlisib (Novartis)		Copanlisib (BAY 80–6946) PI3Kδ/α inhibitor
Dual PI3K/mTOR inhibitor	GSK458/Omipalisib(GlaxoSmithKline) P7170 (Piramal) SB2343/VS-5584 (Verastem)	BEZ235/Dactolisib (Novartis) GDC-0084 (Novogen) GDC-0980/Apitolisib (Genentech) LY3023414 (Eli Lilly) PQR309/Bimiralisib (PIQUR Therapeutics) XL765/Voxtalisib (Sanofi) SF-1126 (SignalRx)	PF-05212384/gedatolisib/PKI-587 (Pfizer)		
Others		CUDC-907/Fimepinostat (Curis)	Rigosertib/ON-01910 (Onconova Therapeutics)		

Yang et al. (2019)

ultimately promoted T cell activation and CD8$^+$ T cell-mediated cytotoxicity via increased expression of granzyme B (De Henau et al. 2016; Horwitz et al. 2018).

Another potent, highly selective, and reversible pan-class I PI3K inhibitor, Copanlisib (BAY 80–6946), with predominant activity against the p110α and p110δ isoforms is currently in clinical development. A recent study showed a better clinical outcome despite unaltered tumor-infiltrating CD8$^+$ T cell numbers (Munoz et al. 2021). This suggests that PI3Kα/δ inhibition may have antitumor effects that are independent of the CD8$^+$ T cell compartment. Taselisib (GDC-0032) is a potent and selective inhibitor of p110α, p110δ, and p110γ PI3K, with less potency towards the p110β isoform. A phase I trial of taselisib reported a 36% response rate in breast cancer patients with *PIK3CA* mutant tumors compared to 0% response in patients whose tumors lacked *PIK3CA* mutations (Tamura et al. 2018). In a phase II trial of Fulvestrant plus Taselisib, patients with mutant *PIK3CA*-expressing breast cancer had substantially better overall responses than wild-type *PIK3CA* tumors (Tamura et al. 2018). A phase III study utilizing taselisib plus fulvestrant versus placebo plus fulvestrant in patients with metastatic breast cancer had no clinical utility given its safety profile and modest clinical benefit (NCT02340221) (Dent et al. 2021). Concerning dual PI3K/mTOR inhibitors, Dactolisib (BEZ235) is currently in phase I/II clinical trials. FDA terminated a phase I trial of BEZ235 in advanced renal cell carcinoma due to frequent dose-limiting toxicities. In a phase II trial, 29 of 31 patients discontinued treatment due to adverse events and the primary progression-free survival (PFS) endpoint was not met, resulting in trial termination (Carlo et al. 2016). Another pan-PI3K/mTOR inhibitor, Apitolisib (GDC-0980) showed serious adverse events in a phase II trial in patients with renal cell carcinoma, which led to discontinuation of the trial (Fruman et al. 2017).

4 Perspectives

CD8$^+$ T cells are responsive to multiple extracellular signals that can influence the quantity and quality of memory CD8$^+$ T cells, determining the outcome of the immune response. The PI3K signaling pathway plays a significant role in T cell migration, proliferation, survival, and memory generation, making PI3K inhibition an attractive target for cancer immunotherapy. This chapter discussed how modulating specific PI3K isoforms and downstream signaling pathways might control distinct facets of effector and memory CD8$^+$ T cell differentiation. After more than three decades from the discovery of PI3K activity, an understanding of the role of p110δ in T cell function, differentiation, and memory generation turned out to be a critical point in the development of PI3K targeting drugs. However, clinical trials testing PI3Kδ inhibitors led to impaired CD8$^+$ T cell responses during infection, and these trials are on hold. This issue of induction of adverse events by systemic administration of isoform-specific PI3Kδ inhibitors can be overcome by utilizing adoptive cell transfer (ACT) therapy. As shown by Abu Eid et al. inhibition of PI3Kδ in CD8$^+$ T cells ex vivo, before their adoptive transfer into B16 tumor-bearing mice, led to a

delay in the tumor growth, in the terminal differentiation of these cells and promoted their therapeutic activity (Abu Eid et al. 2017). This suggests that modulation of PI3K and its downstream targets might be a promising approach to improve cellular therapies such as Chimeric Antigen Receptor (CAR) T cell immunotherapy and engineered T Cell Receptor (TCR) therapy. PI3K inhibition during CAR-T manufacturing and ex vivo expansion restored the CAR-T effector/memory ratio and impacted effector T cell development, memory formation, and cytokine production in tumor-specific therapeutic $CD8^+$ T cells, leading to an efficient antitumor response (Abu Eid et al. 2017; Aragoneses-Fenoll et al. 2018; Zheng et al. 2018). A better understanding of the relationship between PI3K signaling and the role of regulation of its isoforms in T cell biology can enlighten the development of new therapies and help improve our fundamental understanding of T cell biology.

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PI3K Isoforms in B Cells

Elissa K. Deenick, Julia Bier, and Anthony Lau

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Abstract Phosphatidylinositol-3-kinases (PI3K) control many aspects of cellular activation and differentiation and play an important role in B cells biology. Three different classes of PI3K have been described, all of which are expressed in B cells. However, it is the class IA PI3Ks, and the p110δ catalytic subunit in particular, which seem to play the most critical role in B cells. Here we discuss the important role that class IA PI3K plays in B cell development, activation and differentiation, as well as examine what is known about the other classes of PI3Ks in B cells.

Abbreviations

AID	Activation-induced cytidine deaminase protein
BCR	B cell receptor
DZ	Dark zone

E. K. Deenick (✉) · J. Bier · A. Lau
Garvan Institute of Medical Research, Darlinghurst, NSW, Australia
e-mail: e.deenick@garvan.org.au

E. K. Deenick
Faculty of Medicine and Health, UNSW, Sydney, Australia

J. Bier · A. Lau
St Vincent's Clinical School, Faculty of Medicine and Health, UNSW, Sydney, Australia

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FOXO	Forkhead box O
GCs	Germinal centres
GOF	Gain of function
Ig	Immunoglobulin
LOF	Loss of function
LZ	Light zone
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
MZ	Marginal Zone
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol-3-kinase
PI-(4,5)-P_2	Phosphatidylinositol-(4,5)-biphosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
RAG1	Recombination-activating genes 1
RAG2	Recombination-activating genes 2
SHIP	SH2-domain containing inositol-5-phosphatases
TD	T dependent
TI	T independent

1 Introduction

The phosphatidylinositol-3-kinase (PI3K) family of proteins are lipid kinases that phosphorylate the 3' hydroxyl group of the inositol ring of phosphatidylinositol lipids, allowing them to regulate cellular processes critical for activation and differentiation. Eight PI3K isoforms have been characterised and are divided into three classes (classes I-III) (Bilanges et al. 2019). Class I PI3Ks are the principal isoforms involved in B cell signalling as discussed in detail below. However, many of the other classes of PI3K are also expressed in immune cells, including B cells (Okkenhaug 2013).

2 Class I PI3K

Class I PI3Ks are heterodimers composed of a catalytic subunit and a regulatory subunit and mediate the phosphorylation of phosphatidylinositol-(4,5)-biphosphate (PI-(4,5)-P_2) to phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3). Class I PI3Ks are further categorised into IA and IB. The class IA PI3Ks heterodimers are comprised of a p110α, p110β or p110δ catalytic subunit (encoded by *PIK3CA, PIK3CB* and *PIK3CD*, respectively) bound by one of several possible regulatory subunits: p85α, p85β, p55α, p55γ, or p50α (commonly referred to collectively as p85) (Bilanges et al.

2019; Okkenhaug 2013; Fruman et al. 2017). The class IB PI3K is a heterodimer composed of the p110γ catalytic subunit (encoded by *PIK3CG*) and either the p101 (*PIK3R5*) or p84 (*PIK3R6*) regulatory subunit (Okkenhaug 2013; Fruman et al. 2017).

2.1 Class IA PI3K

2.1.1 Activation and Signalling Through Class IA PI3K in B Cells

Class IA PI3Ks are activated downstream of multiple cell surface receptors that are expressed on B cells (Fig. 1), including the B cell antigen receptor (BCR), CD40, CD19, toll-like receptors, cytokine receptors and BAFFR (Ren et al. 1994; Attridge et al. 2014; Jellusova et al. 2013; Jellusova and Rickert 2016; Okkenhaug et al. 2007; Dil and Marshall 2009). Upon receptor ligation, PI3K is activated and phosphorylates membrane PI-(4,5)-P2 to generate PIP_3, which acts as a binding site for a number of signalling molecules, enabling signal propagation of multiple downstream pathways. PIP_3 levels are regulated by the lipid phosphatases SH2 domain-containing inositol-5-phosphatases (SHIP) and phosphatase and tensin homolog deleted on chromosome ten (PTEN), which dephosphorylate PIP_3 to generate PI-(3,4)-P_2 and PI-(4,5)-P_2, respectively, inhibiting signal transduction (Bilanges et al. 2019; Okkenhaug 2013).

Downstream signalling proteins are recruited to PIP_3 through interactions with their pleckstrin-homology domains. Key proteins that are recruited to PIP_3 include phosphoinositide-dependent kinase-1 (PDK1), and the serine/threonine kinase AKT (protein kinase B), a major mediator of PI3K signalling (Limon and Fruman 2012). PDK1 phosphorylates a threonine residue in the activation loop of AKT (Thr 308), which is required for its activation. For maximal activation, AKT is also phosphorylated at the serine residue Ser 473, located in the C-terminal hydrophobic motif, by mechanistic target of rapamycin complex 2 (mTORC2) (Limon and Fruman 2012; Manning and Toker 2017). AKT is a major effector of the PI3K pathway, with many AKT substrates linked to cell function and disease reported thus far (Manning and Toker 2017).

One key target of AKT in B cells is the Forkhead Box subgroup O (FOXO) family of transcription factors (consisting of FOXO1, FOXO3a, FOXO4 and FOXO6). These proteins can mediate both the activation and repression of transcription and thus control multiple processes in B cells including proliferation, differentiation and survival as discussed further below (Zaiss and Coffer 2018). AKT phosphorylates FOXO proteins leading to their nuclear exclusion and degradation and inhibiting their transcriptional activity (Limon and Fruman 2012; Zaiss and Coffer 2018).

Activation of AKT also leads to the activation of mechanistic targets of rapamycin complex 1 (mTORC1). In turn, mTORC1 activates downstream pathways that promote protein and lipid synthesis, and are critical for cell growth, metabolism and differentiation (Limon and Fruman 2012; Iwata et al. 2017).

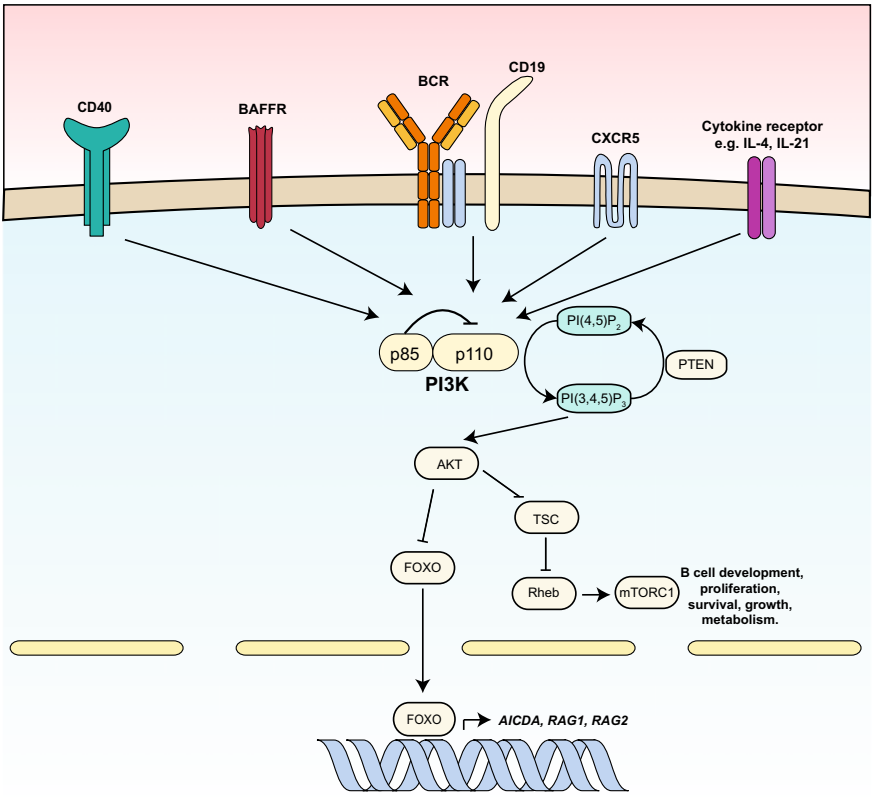

Fig. 1 Class I PI3K signalling pathways in B cells. The schematics show a simplified class IA PI3K signalling pathway in B cells where PI3K can be activated downstream of different cell surface receptors (e.g. BCR, CD40, IL-4, IL-21). Once activated, PI3K phosphorylates $PI(4,5)P_2$ into $PI(3,4,5)P_3$, leading to the activation of AKT. This in turn results in the phosphorylation of the transcription factor FOXO, leading to its exclusion from the nucleus. This inhibits the FOXO-mediated transcription of genes such as *AICDA*, *RAG1* and *RAG2*. AKT can also activate mTORC1 via phosphorylation and inhibition of TSC2. mTORC1 regulates multiple downstream pathways in B cells such as proliferation, survival, growth and metabolism

2.1.2 The Role of Class IA PI3K in B Cells

Research in the past few decades has revealed that p110δ is the PI3K isoform most central to regulating B cell behaviour. Roles for p110δ in development, activation and differentiation of B cells have been demonstrated through the use of multiple approaches, including pharmacological inhibitors, as well as mouse models with loss of expression of p110δ (Clayton et al. 2002; Jou et al. 2002), or expression of a catalytically inactive form of p110δ ($p110\delta^{D910A}$) (Okkenhaug et al. 2002). In addition, mice that lack the corresponding regulatory subunit p85α have been also generated (Suzuki et al. 1999; Donahue et al. 2004). As p85α stabilises p110δ,

loss of p85α also results in reduced p110δ levels. Conversely, mouse models that increase PI3K signalling such as deficiency of PTEN (which negatively regulates PI3K activity) (Suzuki et al. 2003; Anzelon et al. 2003) and mutations that result in p110δ gain of function (GOF) have been also studied (Avery et al. 2018; Preite et al. 2018; Stark et al. 2018; Wray-Dutra et al. 2018). Together these approaches, as well as the discovery of patients who have mutations affecting the PI3K signalling pathway, have helped define the non-redundant role of p110δ in B cells as discussed below.

2.1.3 B Cell Development

B cell development occurs in the bone marrow where emerging B cells assemble a functional BCR in a step-wise manner (Fig. 2). This begins at the pro-B cell stage with rearrangement of the V_H, D_H and J_H gene segments of the immunoglobulin (Ig) gene locus to form the heavy chain variable region, in a process that requires recombination-activating genes 1 (RAG1) and RAG2 (Schatz and Ji 2011). The newly rearranged Igμ chain is then expressed on the cell surface, together with a surrogate light chain, forming the pre-BCR (Clark et al. 2014; Winkler and Mårtensson 2018). Signalling from the pre-BCR is an important checkpoint to ensure that only B cells with a productively rearranged heavy chain proceed through B cell development. Signalling through the pre-BCR results in proliferation and subsequent V_L and J_L recombination to form the Ig light chain, which then combines with Igμ to produce a mature BCR (Clark et al. 2014; Winkler and Mårtensson 2018). Signalling through this newly formed BCR is important for further selection of the maturing B cell.

Given that PI3K is activated downstream of both the pre-BCR and BCR, it was reasonable to speculate that p110δ may play a role in B cell development in the bone marrow. Subsequent studies of mice with disrupted p110δ signalling revealed a fairly mild defect in B cell development with a relative increase in pro-B cells and a decrease in more mature cells (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002). A similar pattern was observed in mice deficient for the regulatory subunit p85α (Suzuki et al. 1999; Fruman et al. 1999).

The relatively mild B cell developmental defect in p110δ loss of function (LOF) mice may result from redundancy with the other PI3K catalytic subunits, namely p110α and p110β. Consistent with this, loss of function of p110α combined with $p110\delta^{D910A}$ results in more severe defects in B cell development than in mice with $p110\delta^{D910A}$ alone (Okkenhaug et al. 2002; Ramadani et al. 2010). Specifically, these mice had a block in B cell development at the pre-B cell stage, resulting in dramatically reduced pre-B cells and a 95% reduction in immature and mature B cell numbers in the bone marrow compared to WT mice (Ramadani et al. 2010). Deficiency of p110α alone had no effect on B cell development. Similarly, p110β deficiency had no effect on B cell development either on its own or combined with $p110\delta^{D910A}$ (Ramadani et al. 2010). This is consistent with the low levels of p110β observed in B cells (Janas et al. 2008).

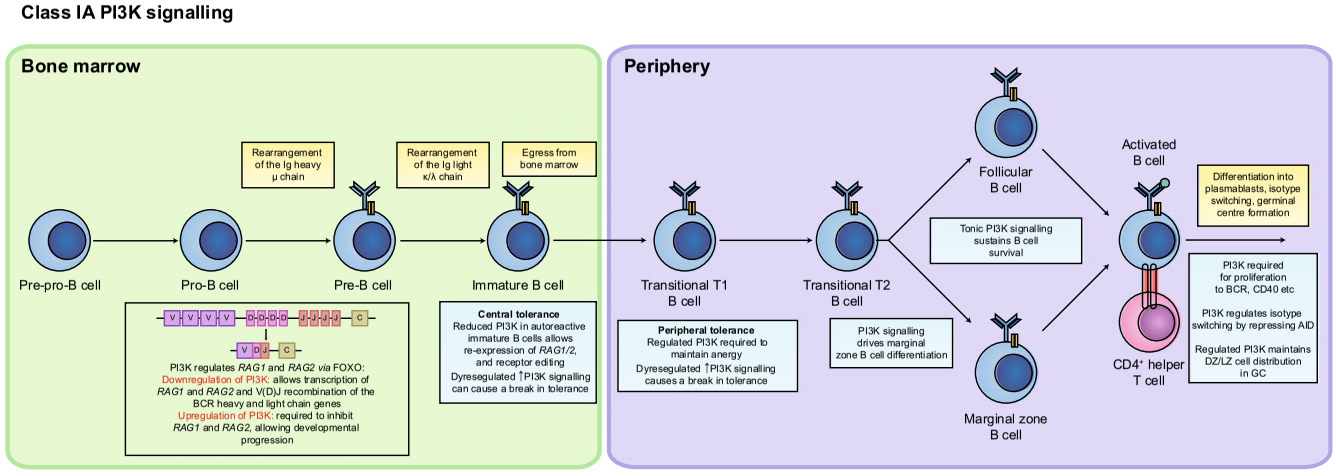

Fig. 2 Class I PI3K signalling in B cell development and activation. B cells begin development in the bone marrow, undergoing rearrangement of the immunoglobulin (Ig) heavy chain, followed by rearrangement of the light chain, resulting in expression of the mature BCR. Immature B cells then migrate to the periphery, undergo successive transitional stages and mature into follicular or marginal zone B cells. During development, B cell tolerance is maintained by both central and peripheral tolerance mechanisms. Upon BCR stimulation and appropriate signals from $CD4^+$ helper T cells, B cells become activated, allowing for secretion of antibodies or generation of immunological memory. Class IA PI3K signalling in B cells, particularly through the p110δ and to a lesser extent the p110α catalytic subunits, is required for cell differentiation, proliferation and survival. Furthermore, regulation of the PI3K pathway is critical to maintain developmental homeostasis and tolerance checkpoints. Dysregulated PI3K activity compromises multiple stages of B cell development and tolerance, resulting in immunodeficiency and autoimmunity

There are several possible explanations for this block in B cell development. As discussed above, PI3K activates AKT which in turn phosphorylates FOXO proteins, leading to their nuclear exclusion and degradation (Zaiss and Coffer 2018). As FOXO proteins can directly activate transcription of *Rag1* and *Rag2* (Amin and Schlissel 2008; Dengler et al. 2008; Herzog et al. 2008), failure to inhibit FOXO activity due to defective PI3K signalling would allow sustained RAG activity preventing further development. Consistent with this, pro-B or immature B cells with disrupted class I PI3K signalling have higher levels of RAG1 and RAG2 than wild-type cells (Ramadani et al. 2010; Verkoczy et al. 2007; Llorian et al. 2007). Further, FOXO1 has been also reported to repress Pax5, a transcription factor that is critical to the commitment of cells to the B cell lineage, and inhibition of p110δ was shown to lead to decreased Pax5 expression via increased FOXO1 (Abdelsamed et al. 2011). Thus, dysregulation of these and other FOXO targets may prevent further development of pro-B cells.

Signalling through both the pre-BCR and the IL-7R is also important for the proliferation and survival of developing murine B cells (Rickert 2013). Thus, disruption of PI3K signalling downstream of these receptors is also likely to impact proliferation and survival. Consistent with this, p110α/p110δ double-deficient B cells were shown to have reduced proliferation in response to IL-7 (Ramadani et al. 2010). Further, disruption of mTORC1 signalling in B cells also leads to a block in early B cell development that is associated with reduced proliferation, increased cell death and dysregulated cellular metabolism (Iwata et al. 2017, 2016). This suggests PI3K-mTORC1 signalling is critical to sustain the proliferation and survival of developing B cells.

In the periphery, PI3Kδ LOF mice had an approximately 50% reduction in B cells, particularly in the marginal zone (MZ) and peritoneal B1 compartments (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002; Ramadani et al. 2010), and analysis of mice with p110δ deletion specifically in B cells (*B*-p110δ$^{fl/fl}$) revealed these defects were B cell-intrinsic (Rolf et al. 2010). Interestingly, p85α$^{-/-}$ mice exhibited a decrease in follicular B cells as well as in the MZ and B1 B cells (Donahue et al. 2004), suggesting that other p110 isoforms affected by the loss of p85α may play a role in follicular B cell development. Indeed, mice lacking both p110δ and p110α activity were subsequently shown to have an even more severe decrease in mature MZ and follicular B cells in the periphery (Ramadani et al. 2010).

Once again, several different pathways downstream of PI3K likely account for these changes. First, it likely reflects a role for PI3K in cell survival. It is known that tonic BCR signalling is required for the survival of B cells in the periphery, and that lack of this tonic signalling can be compensated for by a constitutively active p110α or PTEN deficiency (Srinivasan et al. 2009). Further, cells deficient in both p110α and p110δ were found to have increased apoptosis (Ramadani et al. 2010). This is probably due, at least in part, to a failure to downregulate FOXO activity in the absence of PI3K signalling, thus allowing the transcription of genes such as *BCL2L11* (which encodes the pro-apoptotic molecule Bim) (Srinivasan et al. 2009).

However, expression of a Bcl-2 transgene in p85α$^{-/-}$ mice restored follicular B cell numbers but failed to restore MZ and B1 B cell numbers (Donahue et al. 2004; Janas et al. 2008). This indicates that PI3K plays a critical role not only in the survival

of these cells but also in their differentiation. Mice deficient in AKT1/2 also show decreased MZ cells (Calamito et al. 2010), while mice deficient in FOXO1 have increased MZ cells (Chen et al. 2010). This suggests that a PI3K-AKT is needed to suppress FOXO1 and allow MZ differentiation. This process is also known to be dependent on NOTCH2 signalling (Pillai and Cariappa 2009), suggesting that FOXO1 may regulate NOTCH2 signalling. Indeed, a recent paper reported that FOXO1 could suppress transcription of ADAM28 in B cells, thus preventing cleavage of NOTCH2 and inhibiting MZ development (Zhang et al. 2017).

2.1.4 Effects of Excessive PI3K Signalling on B Cell Development

It is clear that B cells require PI3K signalling–particularly through p110δ—for proper development and differentiation. However, studies have also demonstrated that PI3K must be tightly controlled, as excessive PI3K also leads to dysregulated B cells. Two different models have given insight into the effects of excessive PI3K signalling.

As PTEN converts PIP_3 to PIP_2, deficiency of PTEN results in increased levels of PIP_3, mimicking increased PI3K activation (Fruman et al. 2017; Anzelon et al. 2003). Mice lacking PTEN in their B cells ($b\text{Pten}^{fl/fl}$) have increased peritoneal B1 cells as well as increased splenocytes, particularly transitional and MZ B cells (Suzuki et al. 2003; Anzelon et al. 2003). Clearly, PTEN deficiency will affect PIP_3 generation, not only by p110δ, but also by other p110 isoforms. Thus, it is not surprising that p110δ deletion only partially corrects the defects seen in PTEN-deficient B cells (Janas et al. 2008).

Mice that express a GOF mutation in p110δ have also been generated (Avery et al. 2018; Preite et al. 2018; Stark et al. 2018; Wray-Dutra et al. 2018), providing a clearer insight into the effects of overactive p110δ specifically, rather than the general increase in class I PI3K signalling. Similar to PTEN-deficient B cells, p110δ GOF mice had increased number and proportion of MZ and B1 cells, at the expense of follicular B cells. The distribution of transitional B cells was also altered, with a higher proportion of T1 cells in p110δ GOF mice, consistent with a developmental block (Avery et al. 2018; Preite et al. 2018; Stark et al. 2018; Wray-Dutra et al. 2018). This increase in B1 and MZ cells was the opposite of what was observed in p110δ-deficient B cells, demonstrating that the level of p110δ signalling controls the differentiation of MZ and B1 cells.

Surprisingly, analysis of the bone marrow of p110δ GOF mice revealed decreased total B cells associated with a block in B cell development between the pro-B and pre-B cell stages and a reduction of mature recirculating B cells (Avery et al. 2018; Stark et al. 2018; Wray-Dutra et al. 2018), similar to what was observed in p110δ LOF B cells. This p110δ hyperactivation resulted in increased AKT activation and increased phosphorylation of FOXO proteins (Stark et al. 2018), which likely blocked the re-expression of RAG1 and RAG2 required for rearrangement of the light chain (Amin and Schlissel 2008; Dengler et al. 2008; Herzog et al. 2008), thus resulting in inhibition of B cell maturation at the pre-B cell stage.

Collectively, these studies demonstrate that class I PI3K activity must be tightly regulated to allow appropriate B cell development.

2.1.5 B Cell Proliferation

The role of different PI3K isoforms in B cell activation has been also explored. This revealed that p110δ LOF B cells proliferate poorly in vitro in response to various stimuli, including αIgM, αCD40 and IL-4 (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002). Pharmacological interference also revealed a dose-dependent decrease in proliferation in response to the p110δ-specific inhibitor, IC87114 (Bilancio et al. 2006). This lack of proliferation was associated with a failure to induce cell cycle machineries such as cyclin D2 and A (Bilancio et al. 2006). However, some of this effect may be secondary to decreased survival, as increased apoptosis was also observed in the absence of either the catalytic subunit p110δ or the regulatory subunit p85α (Clayton et al. 2002; Suzuki et al. 1999; Fruman et al. 1999). In contrast, B cells deficient for p110α displayed normal proliferation in response to αIgM, αCD40, IL-4 or LPS (Ramadani et al. 2010), again demonstrating that p110δ is the primary class I PI3K involved in B cell signalling.

2.1.6 Antibody Production

The key role of B cells is the production of protective antibodies, and PI3K also plays a critical role in this function. Unimmunised p110δ LOF mice were found to show generally reduced levels of serum Ig, particularly IgM, but also variably IgG subclasses and IgA, depending on the mouse model (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002). Mice deficient in both p110δ and p110α have even lower levels of serum IgM and IgG (Ramadani et al. 2010). Antibody responses to both T dependent (TD) and T independent (TI) antigens were also compromised in p110δ LOF mice, with responses to TD antigens most affected (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002; Zhang et al. 2008). The compromised TD antibody responses were associated with dramatically decreased or absent germinal centres (GCs) in the spleen, lymph nodes and Peyer's Patches (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002).

However, *B*-p110δ$^{fl/fl}$ mice showed no reduction in GC B cell numbers following TD immunisation (Rolf et al. 2010), suggesting a B cell-extrinsic factor in GC development and/or maintenance. Indeed, mice in which p110δ was conditionally knocked out in T cells (*T*-p110δ$^{fl/fl}$), also had reduced GC B cells in response to immunisation, due to a failure to generate the CD4$^+$ T follicular helper cells that are required to support TD B cell responses (Rolf et al. 2010). Furthermore, *T*-p110δ$^{fl/fl}$ mice, but not *B*-p110δ$^{fl/fl}$ mice, showed severely impaired affinity maturation (Rolf et al. 2010). Taken together, these findings reveal that for the generation of GCs and long-lived antibody responses, p110δ is more important in CD4$^+$ T cell function, than in B cell function.

Conversely, unimmunised p110δ GOF mice were observed to have increased GC and plasma cell numbers compared to WT mice, suggesting that PI3K signalling may be a mediator of terminal B cell differentiation (Preite et al. 2018; Wray-Dutra et al. 2018). However, some of these effects may be secondary to defects in CD4$^+$ T cells

(Preite et al. 2018; Wray-Dutra et al. 2018; Bier et al. 2019), or a break in tolerance (discussed below). Indeed, when antigen-specific responses of p110δ GOF B cells were tracked in the presence of WT $CD4^+$ T cells, the GC responses were variably found to be either normal or only slightly increased (Avery et al. 2018; Preite et al. 2018; Wray-Dutra et al. 2018). Thus, the role of p110δ in B cells in driving GC formation is likely to be context-dependent, and reliant on the particular signals the B cell receives during activation. It is clear, however, that p110δ GOF does result in altered distribution of cells within the GC, with an increase in the light zone (LZ) and a decrease in the dark zone (DZ) (Avery et al. 2018; Preite et al. 2018; Wray-Dutra et al. 2018). Within the DZ, FOXO1 is known to be active in the nucleus where it can drive CXCR4 expression. Consistent with this, FOXO1 deletion in B cells has been shown to ablate the DZ (Dominguez-Sola et al. 2015; Sander et al. 2012). Increased PI3K signalling, which usually occurs in the LZ but not the DZ, would thus suppress FOXO1 activity and lead to loss of DZ cells (Dominguez-Sola et al. 2015; Sander et al. 2012; Inoue et al. 2017). Interestingly, this disturbed DZ/LZ balance in GCs of p110δ GOF B cells did not prevent somatic hypermutation or affinity maturation (Avery et al. 2018). In contrast, complete ablation of FOXO1 or expression of a constitutively active p110α were found to suppress somatic hypermutation and the selection of high-affinity cells more strongly (Dominguez-Sola et al. 2015; Sander et al. 2012; Inoue et al. 2017), suggesting that these processes are controlled by a continuum of PI3K/FOXO activity with smaller disturbances allowing these processes to proceed.

2.1.7 Isotype Switching

The levels of different Ig isotypes in serum are regulated not only by the proliferation and differentiation of the B cells but also by their ability to switch from IgM to other downstream isotypes. Multiple lines of evidence support a critical role for class I PI3K in regulating isotype switching. First, genetic ablation of p110δ activity or the use of the p110δ inhibitor IC87114 resulted in increased isotype switching in vitro (Dil and Marshall 2009; Zhang et al. 2008; Omori et al. 2006). This was associated with an increase in activation-induced cytidine deaminase (AID) expression (Zhang et al. 2008; Omori et al. 2006), which is required for isotype switching, as well as an increase in germline transcripts (Zhang et al. 2008), which are a marker of locus accessibility prior to switching.

Second, B cells with increased class I PI3K signalling due to PTEN deficiency or p110δ GOF showed increased IgM production in vivo (Suzuki et al. 2003; Avery et al. 2018; Preite et al. 2018; Wray-Dutra et al. 2018). Furthermore, $b\text{Pten}^{fl/fl}$ or p110δ GOF cells stimulated in vitro showed poor isotype switching (Avery et al. 2018; Janas et al. 2008; Omori et al. 2006). This failure of switching was associated with normal levels of germline transcripts but decreased levels of AID expression (Suzuki et al. 2003; Avery et al. 2018; Janas et al. 2008; Omori et al. 2006; Chen et al. 2015). However, overexpression of AID did not fully rescue isotype switching in $b\text{Pten}^{fl/fl}$ (Omori et al. 2006), suggesting that other factors also contributed to this defect. Interestingly, p110δ deficiency does not fully restore isotype switching in PTEN-deficient

B cells, however PI3K inhibitors against p110α or p110δ can increase switching, suggesting that both p110α and p110δ are responsible for inhibiting isotype switching in these systems (Janas et al. 2008; Chen et al. 2015).

Again, this effect of class I PI3K on isotype switching is probably controlled via FOXO1, as FOXO1-deficient B cells show a similar defect in switching (Dengler et al. 2008; Dominguez-Sola et al. 2015; Sander et al. 2012). This is thought to result from a requirement for FOXO1 in controlling AID expression and/or a role for FOXO1 in targeting AID to Ig switch regions (Dominguez-Sola et al. 2015; Sander et al. 2015). Thus, regulation of PI3K is required for AID-mediated class switch recombination and effective antibody responses.

2.1.8 B Cell Tolerance

During B cell development in the bone marrow, a large number of self-reactive B cells are generated due to the random nature of the recombination of the BCR (Wardemann et al. 2003). Thus, mechanisms are in place to eliminate these self-reactive B cells and prevent them from becoming activated and producing pathogenic autoreactive antibodies. The first of these, known as central tolerance, occurs in the bone marrow following rearrangement and expression of the BCR. If strong receptor signalling occurs at this point, indicating strong binding to self-Ag present in the bone marrow, the B cell will undergo receptor editing or apoptosis (Nemazee 2017). Receptor editing involves ongoing recombination of the Ig gene locus, leading to expression of a new BCR. Tolerance mechanisms also exist in the periphery to deal with cells that escape central tolerance. Thus, self-reactive cells that make it to the periphery can become anergised by encounter with self-antigen, a state characterised by desensitisation of the BCR and decreased lifespan (Goodnow et al. 2005). Tolerance mechanisms also exist in the GC to silence self-reactive cells that are generated during somatic hypermutation in the GC (Brink and Phan 2018).

Class I PI3Ks play a role in multiple stages of B cell tolerance. It has been demonstrated that B cells that have increased PI3K signalling due to deletion of *Pten*, or expression of a constitutively active p110α, are able to escape central tolerance even when their BCR has high affinity for self-antigen (Greaves et al. 2019; Cheng et al. 2009). This involves two mechanisms: the suppression of FOXO-induced RAG1/2 expression, which prevents receptor editing, and the increased survival of self-reactive cells (Greaves et al. 2019; Cheng et al. 2009).

In models of self-reactivity leading to anergy, the level of PI3K signalling was also shown to be important. Anergic B cells in both mice and humans have been described to have increased levels of PTEN (Browne et al. 2009; Smith et al. 2019) or SHIP-1 activation (O'Neill et al. 2011), depending on the type of antigen the BCR recognises. As PTEN and SHIP-1 dephosphorylate PIP_3, they both negatively regulate signalling downstream of class I PI3K. The important role of these two phosphatases in maintaining anergy is demonstrated by the finding that deletion of either PTEN or SHIP-1 can break anergy and allow activation of self-reactive cells to produce autoreactive antibodies (Browne et al. 2009; O'Neill et al. 2011;

Akerlund et al. 2015; Setz et al. 2019; Getahun et al. 2016). Similarly, self-reactive p110δ GOF B cells also escaped anergy and became activated, forming autoantibody secreting plasmablasts and GCs (Lau et al. 2020). Interestingly, B cells within these self-reactive p110δ GOF GCs underwent somatic hypermutation; however, there was selection for cells that lost affinity for the self-antigen and not for high-affinity clones, indicating that tolerance within the GC was maintained (Lau et al. 2020). Together, these studies demonstrate that the strength of signalling downstream of class I PI3Ks is important for central tolerance and the induction and maintenance of anergy, but is not required for tolerance within the GC.

2.1.9 Class IA PI3Ks in Human B Cells

A handful of patients have been also identified with biallelic LOF mutations in *PIK3CD* or *PIK3R1* (Sogkas et al. 2018; Cohen et al. 2019; Sharfe et al. 2018; Conley et al. 2012; Tang et al. 2018). Like p110δ- or p85α-deficient mice, these patients also show variably decreased B cell numbers, consistent with altered B cell development/survival, and a lack of functional B cell responses, indicated by decreased immunoglobulin levels and memory B cells (Sogkas et al. 2018; Cohen et al. 2019; Sharfe et al. 2018; Conley et al. 2012; Tang et al. 2018).

Similarly, patients with GOF mutations in *PIK3CD*, or LOF in *PIK3R1*, have also been described (Lucas et al. 2014a, b; Deau et al. 2014; Angulo et al. 2013). In both cases, this leads to increased p110δ activation, either through directly increasing catalytic activity (in the case of *PIK3CD* mutations) or indirectly by reducing the inhibitory function of the regulatory subunit (in the case of *PIK3R1* mutations). These patients also present with defects in B cell development, a lack of functional B cell responses associated with decreased memory B cells, and defective isotype switching (Avery et al. 2018; Lucas et al. 2014a; Angulo et al. 2013; Elkaim et al. 2016; Petrovski et al. 2016; Dulau Florea et al. 2017; Wentink et al. 2017). Importantly, these patients also have increased rates of autoimmunity and autoreactive B cells, consistent with a break in B cell tolerance (Lau et al. 2020; Elkaim et al. 2016; Tangye et al. 2019; Coulter et al. 2017; Maccari et al. 2018). Thus, class I PI3Ks play similar roles in human B cells, with the level of PI3K signalling needing to be tightly balanced in order to generate productive antibody responses.

2.1.10 B Cell Malignancies

PI3K signalling is one of the most studied pathways in cancer, and alterations in the PI3K/AKT axis have been associated with multiple B cell malignancies (Fruman and Rommel 2014). For example, mutations that alter the expression or function of the catalytic subunits p110α, p110δ, p85α or PTEN have been described in a range of B cell malignancies including diffuse large B cell lymphoma, Burkitt lymphoma, mantle cell lymphoma and follicular lymphoma (Thorpe et al. 2015; Pfeifer et al. 2013; Chapuy et al. 2018; Love et al. 2012; Zhang et al. 2013; Psyrri et al. 2009). In

addition, patients with increased PI3K signalling due to germline GOF mutations in *PIK3CD* or LOF mutations in *PIK3R1* display increased rates of B cell lymphoma (Elkaim et al. 2016; Coulter et al. 2017; Durandy and Kracker 2020; Kracker et al. 2014). Together, these findings demonstrate the critical role of PI3K signalling in B cell malignancies. Consequently, multiple class I PI3K inhibitors are now in use or in development for treatment of these B cell malignancies (Fruman and Rommel 2014; Thorpe et al. 2015; Keudell and Moskowitz 2019).

2.2 Class IB PI3K

Unlike class IA PI3Ks, which are activated downstream of tyrosine kinases, the class IB PI3K p110γ is activated downstream of G-protein coupled receptors (Hirsch et al. 2000). Mice deficient in p110γ have normal numbers of B cells in the periphery as well as in the bone marrow (Sasaki et al. 2000; Beer-Hammer et al. 2010) and normal proliferative responses to BCR and CD40 stimulation (Sasaki et al. 2000). While p110γ-deficient mice had decreased IgG1 responses to immunisation with TD antigen, this was thought to be secondary to the T cell defects observed in these mice (Sasaki et al. 2000). Moreover, their anti-OVA IgE and IgG1 responses to an OVA sensitisation airway challenge model were found to be normal (Takeda et al. 2009). Together, this data suggested that p110γ does not play a role in B cell development and activation. However, to determine whether the lack of phenotype observed in p110γ-deficient B cells were due to redundancy with class IA PI3Ks, mice doubly deficient in p110δ and p110γ were generated (Beer-Hammer et al. 2010). Studies with these mice suggested that p110γ may play a small role in controlling numbers of mature B cells in the periphery in the absence of p110δ but did not play any cell-intrinsic role in B cell proliferation (Beer-Hammer et al. 2010).

In other immune cells, such as T cells, p110γ is important downstream of chemokine receptors, however, p110γ-deficient mice showed no defect in B cell migration in response to chemokines (Reif and Cyster 2000; Nombela-Arrieta et al. 2004). Rather, it seems that in B cells p110δ, rather than p110γ, is required for signalling downstream of chemokine receptors such as CXCR5 (Reif et al. 2004). One study on malignant B cells suggested that p110γ plays a role in the migration of these cells (Ali et al. 2018), although it remains to be seen whether this property is specific to malignant B cells or whether there may also be a role for p110γ in normal B cell migration under some circumstances.

Two patients with biallelic loss of *PIK3CG* (that encodes for p110γ) have been identified (Takeda et al. 2019; Thian et al. 2020). These individuals presented with reduced serum antibody levels as did the corresponding mouse model (Takeda et al. 2019). However, it is likely that this phenomenon is not B cell-intrinsic, as the patient B cells proliferated and switched normally in vitro (Thian et al. 2020). Thus, the data both from mouse models and patients indicate that p110γ does not play a significant role in the development or activation of normal B cells.

3 Class II PI3K

There are three members of the class II PI3K – PI3K-C2α, PI3K-C2β and PI3K-C2γ encoded in humans by *PIK3C2A*, *PIK3C2B*, *PIK3C2G,* respectively. Unlike class I PI3Ks, no regulatory subunits have so far been described for the class II PI3Ks and they are thought to act as monomers (Bilanges et al. 2019). The class II PI3K catalyse phosphatidylinositol to PI3P and PI4P to $PI(3,4)P_2$.

Both *PIK3C2A* and *PIK3C2B* are expressed in B cells; indeed *PIK3C2B* is expressed more highly in B cells than in any of the other immune cell populations studied (Monaco et al. 2019; Schmiedel et al. 2018). In contrast, *PIK3C2G,* which has much more tissue-restricted tissue expression, is not observed in B cells. Despite the high expression of these molecules in B cells, little is known about the role of either PI3K-C2α or PI3K-C2β in B cell biology. Mice deficient in PI3K-C2α have been generated and were found to be embryonically lethal, therefore the immune effects of PI3K-C2α have not been studied (Bilanges et al. 2019). Interestingly, one mouse model was developed that expressed a hypomorphic PI3K-C2α. These mice developed kidney disease with immune complexes and had increased spleen cellularity, including B cells and increased serum IgM and IgA. However, bone marrow chimeras demonstrated that this effect was not intrinsic to the immune cells (Harris et al. 2011). Further, five patients with LOF mutations in *PIK3C2A* have recently been described. These patients were not reported to have a clinical phenotype suggestive of immunodeficiency, however, their immune system was not examined (Tiosano et al. 2019). Together, these results suggest that PI3K-C2α is not critical for B cell development and function but this remains to be definitively demonstrated.

Similarly, mice have been generated in which PI3K-C2β is knocked out or kinase-dead. These mice are healthy and viable (Harada et al. 2005; Alliouachene et al. 2015). Further, a role for PI3K-C2β in $CD4^+$ T cell and mast cell activation through regulation of the K^+ channel KCa3.1 has been demonstrated (Srivastava et al. , 2012, 2017, 2009), however, once again the development of B cells has not been investigated, although the very high levels of expression of *PIK3C2B* in B cells suggest it is likely to play a role in their biology. Thus, more work is required to understand the potential role of PI3K-C2α, PI3K-C2β in B cell development and function.

4 Class III PI3K

Vps34 is the only member of the class III PI3Ks. It is encoded by *PIK3C3* and converts phosphatidylinositol into phosphatidylinositol-3-phosphate. Vps34 functions in different subcellular compartments where it regulates processes such as phagocytosis, endocytic trafficking and autophagy (Bilanges et al. 2019; Backer 2016). Vps34 works in complex with Vps15 (encoded by *PIK3R4*) and Beclin-1.

While several studies have assessed the role of Vps34 in other immune populations such as T cells (McLeod et al. 2011; Parekh et al. 2013; Willinger and Flavell 2012),

the role of Vps34 in B cells remains largely unknown. However, given that the genes encoding Vps34, Vps15 and Beclin-1 are all expressed in B cells (Monaco et al. 2019; Schmiedel et al. 2018), it is likely that Vps34 does play some role in regulating B cell behaviour, however, further analysis of Vps34 deficient B cells will be required to determine this.

5 Conclusion

Current evidence indicates that the class IA catalytic subunit p110δ is the primary PI3K isoform involved in controlling B cell behaviour. Further, data demonstrate that the level of p110δ signalling must be tightly controlled to ensure appropriate B cell development, activation and differentiation, in order to generate protective antibody responses while avoiding harmful autoimmunity. However, other PI3K classes remain poorly understood in B cells and further studies are required to determine if these isoforms play a role in B cell biology.

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PI3K Isoform Signalling in Platelets

Waltraud C. Schrottmaier, Marion Mussbacher, Manuel Salzmann, Julia B. Kral-Pointner, and Alice Assinger

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W. C. Schrottmaier · A. Assinger (✉)
Department of Vascular Biology and Thrombosis Research, Centre of Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria
e-mail: alice.assinger@meduniwien.ac.at

W. C. Schrottmaier
e-mail: waltraud.schrottmaier@meduniwien.ac.at

M. Mussbacher
Department of Pharmacology and Toxicology, University of Graz, Graz, Austria
e-mail: marion.mussbacher@uni.graz.at

Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, USA

M. Salzmann · J. B. Kral-Pointner
Division of Cardiology, Department of Internal Medicine II, Medical University of Vienna, Vienna, Austria
e-mail: manuel.salzmann@meduniwien.ac.at

J. B. Kral-Pointner
e-mail: julia.pointner@meduniwien.ac.at

J. B. Kral-Pointner
Ludwig Boltzmann Institute for Cardiovascular Research, Vienna, Austria

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Abstract Platelets are unique anucleated blood cells that constantly patrol the vasculature to seal and prevent injuries in a process termed haemostasis. Thereby they rapidly adhere to the subendothelial matrix and recruit further platelets, resulting in platelet aggregates. Apart from their central role in haemostasis, they also kept some of their features inherited by their evolutionary ancestor—the haemocyte, which was also involved in immune defences. Together with leukocytes, platelets fight pathogenic invaders and guide many immune processes. In addition, they rely on several signalling pathways which are also relevant to immune cells. Among these, one of the central signalling hubs is the PI3K pathway. Signalling processes in platelets are unique as they lack a nucleus and therefore transcriptional regulation is absent. As a result, PI3K subclasses fulfil distinct roles in platelets compared to other cells. In contrast to leukocytes, the central PI3K subclass in platelet signalling is PI3K class Iβ, which underlines the uniqueness of this cell type and opens new ways for potential platelet-specific pharmacologic inhibition. An overview of platelet function and signalling with emphasis on PI3K subclasses and their respective inhibitors is given in this chapter.

1 Platelet Structure

Mammalian platelets are exceptionally unique cells that, like erythrocytes, lack a nucleus. This distinguishes them strongly from non-mammalian, nucleated thrombocytes (Levin 2013). They derive from megakaryocytes, which extend pro-platelets into the bloodstream and of which platelets eventually fission off (Machlus et al. 2014). Human platelets are roughly 2–5 μm in diameter and circulate the vasculature for about five to nine days (Ghoshal and Bhattacharyya 2014).

Deriving from a megakaryocyte's cytoplasm, their cellular structure is quite different from nucleated cells (Fig. 1a). Platelets contain three major types of granules, a dense tubular system (DTS), an open canalicular system (OCS) and a microtubule coil to maintain their discoid shape. During primary haemostasis platelets massively increase in size while exposing considerable amounts of the OCS' negatively charged phosphatidylserine (PS) to further enhance blood coagulation. The microtubule coil disintegrates during platelet activation, allowing an increase in cell volume and formation of lamellipodia and filopodia via actin polymerisation. The DTS is a remnant of the megakaryocyte's smooth endoplasmic reticulum and serves as site of calcium ion (Ca^{2+}) storage and thromboxane A_2 (TXA_2) synthesis (White 2013). However, the most distinct cellular structures of platelets are three types of granules: dense or δ-granules, lysosomes and α-granules. Dense granules store small molecules like adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, Ca^{2+}, or polyphosphates (Ambrosio and Pietro 2017). Lysosomes comprise glycoside hydrolases like β-hexosaminidase (Holmsen and Dangelmaier 1989). α-granules on the other hand contain over 1000 different proteins, including coagulation factors and inhibitors, pro-inflammatory and pro-atherogenic proteins, adhesion and

membrane proteins, chemokines, antimicrobial proteins and growth factors (Fong et al. 2011).

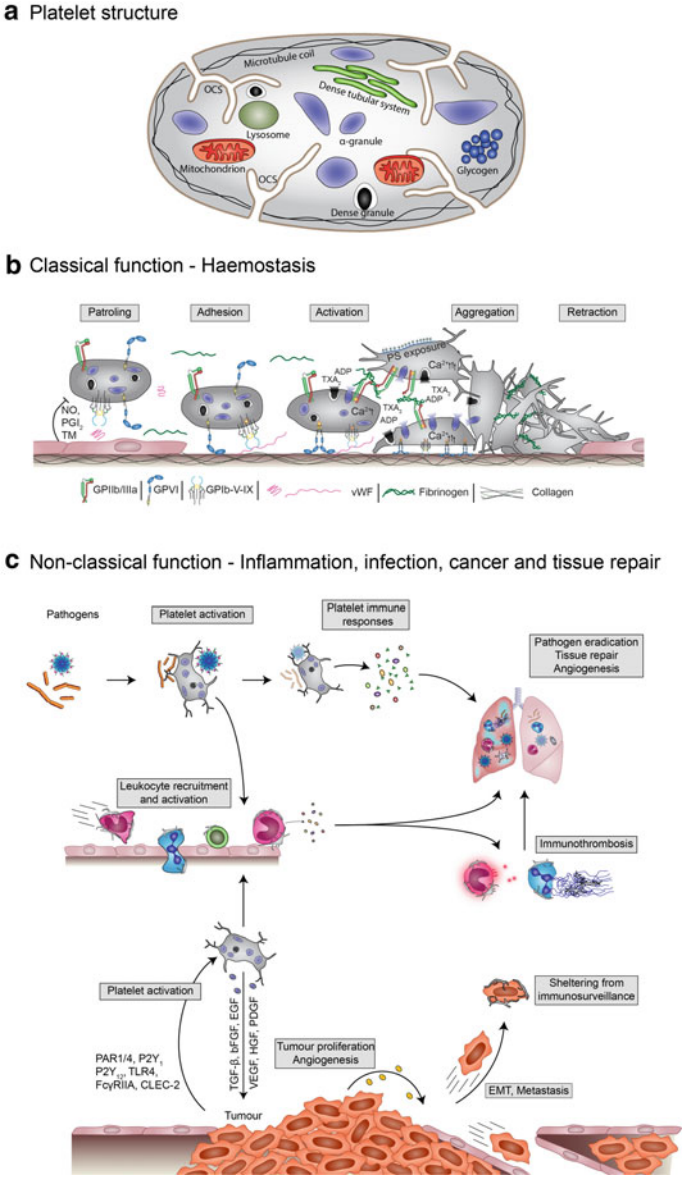
Due to their anucleate state platelets are generally unable to transcribe genetic information into messenger RNA (mRNA). However, platelets do contain different RNA species (e.g. mRNA, microRNA) that are believed to originate from their megakaryocyte precursor and/or plasma uptake (Harrison and Goodall 2008; Gutmann et al. 2020; Best et al. 2017). The RNA content is highest in young platelets (so-called reticulated platelets) and declines over the first 24 hours, enabling the distinction between RNA-positive reticulated platelets (i.e. young) and RNA-negative platelets. Despite containing various mRNAs, splicing and protein translation are rare events in platelets and only a handful of proteins have been shown to be actively de novo synthesized within platelets (e.g. interleukin-1β, B cell lymphoma 3 protein, tissue factor and coagulation factor XI) (Denis et al. 2005; Weyrich et al. 1998; Schwertz et al. 2006; Zucker et al. 2018). Thus, platelets are heavily dependent on their parent megakaryocytes to provide them with the life-long protein repertoire necessary to adequately respond to disturbances in vascular homeostasis, even though most platelets may never need it.

While this concept may seem somewhat inefficient, the presence of large amounts of a myriad of preformed proteins independently of time-consuming de novo synthesis turns these small inconspicuous cells into an armada of ready-to-use cellular Swiss army knives against inflammatory/infectious agents and injuries alike.

From a scientist's perspective, this general lack of protein synthesis also renders platelets very interesting as cellular models to study structural and signalling protein functions without interference due to newly synthesized molecules.

2 The Role of Platelets in Haemostasis and Beyond

In circulation, platelets are kept in a resting state by endothelial cells that constantly release nitric oxide or prostacyclin and express thrombomodulin (Geraldo et al. 2014; Ramackers et al. 2020). Upon vessel injury, circulating, globular von Willebrand factor (vWF) is immobilised on subendothelial collagen and undergoes shear stress-induced conformational unfolding, which strongly enhances its binding affinity to the platelet glycoprotein (GP) Ib-V-IX. This is crucial for initial platelet adhesion, which is further stabilised by binding of collagen to GPVI, inducing a series of signalling cascades that sustain platelet activation. During platelet activation, cytoskeletal proteins cause a conformational change of GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), the most abundant platelet integrin, converting it into its active form in a process called 'inside-out signalling'. Binding of its ligand fibrinogen to GPIIb/IIIa further strengthens adhesion, platelet-platelet interactions and triggers 'outside-in signalling', which leads to spreading, further degranulation, and the release of a variety of soluble agents (Shattil and Newman 2004; Moser et al. 2008). Among these released molecules are dense granule-derived ADP and arachidonic acid-derived thromboxane A_2 TXA_2, which ensure persistent and efficient platelet activation by amplification of the initial


a Platelet structure
Microtubule coil
Dense tubular system
OCS
Lysosome
α-granule
Mitochondrion
OCS
Glycogen
Dense granule
b Classical function - Haemostasis
Patroling
Adhesion
Activation
Aggregation
Retraction
NO, PGI2 TM
PS exposure
ADP
TXA2
Ca2+
GPIIb/IIIa
GPVI
GPIb-V-IX
vWF
Fibrinogen
Collagen
c Non-classical function - Inflammation, infection, cancer and tissue repair
Pathogens
Platelet activation
Platelet immune responses
Pathogen eradication Tissue repair Angiogenesis
Leukocyte recruitment and activation
Immunothrombosis
Platelet activation
PAR1/4, P2Y1, P2Y12, TLR4, FcγRIIA, CLEC-2
TGF-β, bFGF, EGF VEGF, HGF, PDGF
Tumour proliferation Angiogenesis
Sheltering from immunosurveillance
EMT, Metastasis
Tumour

◀**Fig. 1** Platelets are cells with unique structures and diverse functions. **a** Platelets are anucleated cells with characteristic subcellular structures. The open canalicular system (OCS) forms a sponge-like network within the cell which maintains its shape due to a submembranous microtubule coil. Platelets also contain three characteristic types of intracellular granules, α-granules, dense granules and lysosomes, as well as classic intracellular structures such as mitochondria, glycogen storage compartments and an endoplasmic reticulum-like dense tubular system (DTS). **b** In order to maintain haemostasis, circulating platelets constantly patrol the endothelium for injuries. While normally kept in a resting state by endothelium-derived mediators, exposed subendothelial matrix rapidly triggers platelet adhesion via binding of von Willebrand factor (vWF) and collagen. Adherent platelets become activated and release the secondary mediators ADP and thromboxane A_2 (TXA_2) which recruit and further activate platelets in a positive feedback loop. Platelets aggregate, supported by crosslinking via fibrinogen binding, to eventually form a firm thrombus that is stabilised by an insoluble fibrin mesh. Finally, clot retraction reduces the thrombus volume to minimise vessel occlusion. **c** Platelets recognise various invading pathogens, leading to their activation. Activated platelets readily interact with leukocytes to promote their recruitment and activation, including prothrombotic effector functions such as microvesicle release or expulsion of nuclear DNA. Induction of thrombus formation by these immune responses (immunothrombosis) may help to ensnare pathogens. Activated platelets can also launch a direct immune response by releasing intracellular content. Thereby, platelets facilitate pathogen eradication, tissue repair and angiogenesis. Similarly, platelets express numerous receptors that mediate platelet activation and platelet-leukocyte interaction during cancer, subsequently leading to enhanced leukocyte recruitment, activation and immunothrombosis. Additionally, activated platelets release growth factors that act on the tumour and its microenvironment to modulate proliferation and angiogenesis. Platelets also facilitate metastasis by enhancing epithelial-mesenchymal transition (EMT) of tumour cells and by sheltering them from immunosurveillance. Thereby, platelets contribute to progression and malignancy of cancer. TGF-β: transforming growth factor β; bFGF: basic fibroblast growth factor; EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; PDGF: platelet-derived growth factor; PAR: protease-activated receptor, TLR4: Toll-like receptor 4, FcγRIIA: Fcγ receptor IIA; CLEC-2: C-type lectin-like type II

signal. The importance of this positive feedback mechanism becomes apparent in the success of current platelet aggregation inhibitors such as clopidogrel, which antagonises the ADP receptor purinergic receptor $P2Y_{12}$ ($P2Y_{12}$) and aspirin, which blocks cyclooxygenases and therefore inhibits TXA_2 synthesis, indicating that a lack of this amplifying mechanism efficiently prevents thrombus formation (Schrottmaier et al. 2015). Via release of ADP and TXA_2, more and more platelets are recruited to the site of injury. Fibrinogen bound to active GPIIb/IIIa crosslinks adjacent activated platelets and promotes the formation of a firm but reversible platelet aggregate, a 'white thrombus'. In parallel, plasmatic coagulation ('secondary haemostasis') produces large amounts of thrombin, which is not only the key enzyme that cleaves fibrinogen into insoluble fibrin but also a potent activator of protease-activated receptors (PAR) on platelets. Fibrin polymerisation and crosslinking lead to a mesh-like structure, that catches circulating erythrocytes and stabilises the clot into an irreversible fibrin-rich 'red thrombus' that can efficiently close the vessel wound (Licari and Kovacic 2009). To counteract the increased shear stress caused by the resulting decreased vessel lumen, outside-in signalling of platelets induces clot retraction to restore vessel lumen and to support the healing process by diminishing wound surface

(Shattil and Newman 2004; Sorrentino et al. 2015). Platelet function in primary haemostasis is summarised in Fig. 1b.

Besides their essential role in haemostasis, activated platelets rapidly interact with innate immune cells (preferentially monocytes and neutrophils (Ahn et al. 2005)) and exert immunomodulatory functions during inflammation and infection (Fig. 1c). Platelet-leukocyte crosstalk is either achieved by direct cell–cell contact [e.g. by P-selectin (CD62P) binding to P-selectin binding glycoprotein 1 (PSGL-1)] and/or by release of chemokines and cytokines (Schrottmaier et al. 2015; Kral et al. 2016). Direct platelet-leukocyte interaction fosters mutual activation and triggers the release of platelet granule content (including platelet factor 4 (PF4/CXCL4), stromal cell-derived factor 1, soluble CD40 ligand (sCD40L) and sCD62P) and the generation of lipid mediators (e.g. TXA_2), which modulates various leukocyte effector functions (Kral et al. 2016; Gear and Camerini 2003; Grommes et al. 2012). Additionally, platelets themselves act as immune cells by releasing antimicrobial factors such as defensins and immunoglobulins (Yeaman 2014; Schrottmaier et al. 2020). Thereby platelets and platelet-leukocyte interactions are involved in various diseases and contribute to pathogenesis and resolution of inflammatory and infectious diseases.

Being highly responsive and secretory cells, platelets can adapt to changes in tissue homeostasis and initiate regenerative processes of tissue remodelling by promoting cellular migration, proliferation/differentiation, angiogenesis as well as control of apoptosis and cell survival (Fig. 1c) (Eisinger et al. 2018). Mechanistically, these effects are mediated by release of pro-angiogenic [e.g. vascular endothelial growth factor (VEGF)] as well as anti-angiogenic (e.g. thrombospondin) factors (Pavlovic et al. 2016). In addition, platelets modulate tumour progression and metastasis formation by 'hiding' and 'camouflaging' cancer cells from the immune system and decreasing shear forces to 'guide and shelter' cancer cells into the vessel wall (Pavlovic et al. 2016; Stoiber and Assinger 2020). Beyond that, platelets also facilitate early detection of tumours by becoming 'tumour educated', which means that they carry tumour markers and represent promising vectors for targeted drug delivery (Best et al. 2018).

3 Signalling in Platelets

Signalling in platelets can sometimes differ from that of nucleated cells. As there is no nucleus, there are also no transcriptional feedback mechanisms. Nonetheless, platelets express some unexpected molecules and phosphorylation of signalling molecules like extracellular signal-regulated kinases (ERK) or mitogen-activated protein kinase 14 (p38 MAPK) does occur and represents hallmarks of platelet activation and/or degranulation. Furthermore, some pathways that inhibit signal transduction in nucleated cells such as $G_{\alpha i}$, which blocks cyclic adenosine monophosphate (cAMP) signalling and thus cellular responses, promote activation in platelets

instead (Li et al. 2010). A short overview over the most important signalling pathways involved in primary haemostasis and in platelet-mediated immunomodulation is given below.

3.1 Platelet Signalling During Primary Haemostasis

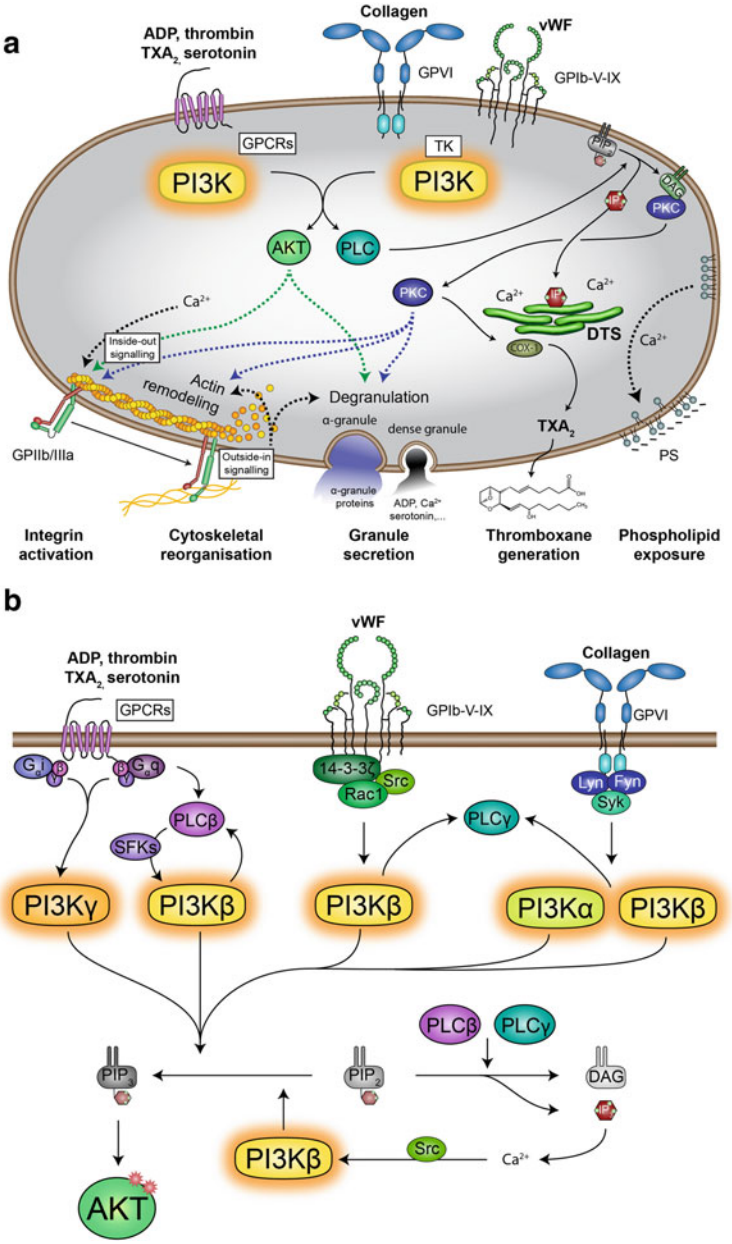
Platelet signalling can be roughly divided into three major pathway axes: phospholipase C (PLC), protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT). The involvement of PI3K in the regulation of key platelet functions is summarised in Fig. 2a.

Binding of vWF to GPIb-V-IX recruits adaptor proteins SRC proto-oncogene (Src), Rac family small GTPase 1 (Rac1) and 14-3-3ζ to trigger PI3Kβ-induced PLCγ activation (Bryckaert et al. 2015). Binding of collagen to GPVI induces also PLCγ activation, but via the Src family kinases (SFK) Fyn proto-oncogene (Fyn) and Lyn proto-oncogene (Lyn), which are brought into vicinity of crosslinked GPVI's immunoreceptor tyrosine-based activation motif (ITAM) regions. This recruits and phosphorylates spleen-associated tyrosine kinase (Syk) that together with PI3Kα or PI3Kβ activates PLCγ (Carrim et al. 2014; Gilio et al. 2009; Kim et al. 2009).

Secreted ADP, TXA_2 and serotonin, as well as thrombin, signal via G-protein-coupled receptors (GPCRs) and are potent activators of platelets. TXA_2 binds to TXA_2/prostaglandin H2 receptor (TP) and serotonin (5-hydroxytryptamin; 5-HT) to $5\text{-}HT_{2A}$ (Offermanns 2006; Djellas et al. 1999). $P2Y_1$ and $P2Y_{12}$ are the two platelet receptors for ADP. While $P2Y_1$ triggers initial platelet aggregation, $P2Y_{12}$ amplifies and preserves platelet activation (Offermanns 2006; Hechler et al. 1998; Storey et al. 2000). The receptors for thrombin in humans are PAR-1 and PAR-4. Most of these GPCRs are coupled to $G_{\alpha q}$ and thereby lead to activation of PLCβ (Offermanns 2006).

PLCβ and PLCγ cleave phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$) to diacylglycerol (DAG) and inositol-(1,4,5)-trisphosphate (IP_3). DAG induces activation of PKC and IP_3 releases Ca^{2+} from the DTS. Increased Ca^{2+} levels in turn result in increased TXA_2 synthesis and granule content release (Li et al. 2010).

PI3K is involved in all major activation pathways. PLCβ, activated through $G_{\alpha q}$ coupled GPCRs, and Ca^{2+} leads to activation of PI3Kβ (Martin et al. 2010) via SFK (Kim et al. 2006) downstream of PLC (Hall et al. 2007; Senis et al. 2014). PLCγ is activated by GPVI via PI3Kα or PI3Kβ (Carrim et al. 2014; Gilio et al. 2009; Kim et al. 2009) and by GPIb-V-IX via PI3Kβ (Bryckaert et al. 2015). $P2Y_{12}$ is coupled to $G_{\alpha i}$ which supports platelet activation via inhibition of cAMP production, although its actual effect on platelet aggregation is rather small. However, $G_{\alpha i}$ signalling releases considerable amounts of $G_{\beta\gamma}$, which in turn target PI3Kγ (Hirsch et al. 2001). PI3K then transforms phosphatidylinositol-(4,5)-bisphosphate ($PI(4,5)P_2$) into phosphatidylinositol-(3,4,5)-trisphosphate ($PI(3,4,5)P_3$) which subsequently activates the central downstream signalling hub AKT. Among the three AKT isoforms, AKT2 is particularly important for platelet function (Woulfe et al. 2004),


a
ADP, thrombin
TXA2, serotonin
Collagen
vWF
GPVI
GPIb-V-IX
GPCRs
TK
PI3K
PI3K
AKT
PLC
PKC
DAG
IP3
Ca2+
Inside-out signalling
Actin remodeling
Degranulation
α-granule
dense granule
DTS
TXA2
PS
GPIIb/IIIa
Outside-in signalling
α-granule proteins
ADP, Ca2+ serotonin,...
Integrin activation
Cytoskeletal reorganisation
Granule secretion
Thromboxane generation
Phospholipid exposure
b
ADP, thrombin
TXA2, serotonin
GPCRs
vWF
GPIb-V-IX
Collagen
GPVI
14-3-3ζ
Src
Rac1
Lyn
Fyn
Syk
PLCβ
PLCγ
SFKs
PI3Kγ
PI3Kβ
PI3Kβ
PI3Kα
PI3Kβ
PIP3
PIP2
DAG
IP3
Ca2+
Src
PI3Kβ
AKT

◀**Fig. 2** Phosphatidylinositol-3-kinase (PI3K) class I isoforms mediate platelet activation in response to various agonists. **a** PI3K is induced by both G protein-coupled receptors (GPCR) and tyrosine kinases (TK). PI3K activity leads to phosphorylation and thus activation of AKT, but can also activate the PLC/Ca^{2+} pathway, both of which signal via a complex interwoven signalling network to mediate essential platelet functions such as activation of the integrin GPIIb/IIIa, cytoskeletal reorganisation, secretion of both α- and dense granule content, TXA_2 generation and phospholipid exposure. **b** Different PI3K isoforms are induced by different platelet receptors. GPCRs, in particular upon ADP stimulation, induce both PI3Kβ and PI3Kγ in dependency of the involved G protein. In contrast, receptors requiring tyrosine kinases signals such as GPIb-V-IX or GPVI activate either only PI3Kβ or both PI3Kα and PI3Kβ. In addition to directly inducing PIP3-mediated AKT activation, PI3K isoforms also stimulate PLCβ and PLCγ which in turn signal via Ca^{2+} and Src kinase to activate PI3Kβ, thereby further augmenting AKT phosphorylation. ADP: adenosine diphosphate; AKT: protein kinase B; COX-1: cyclooxygenase 1; DAG: diacylglycerol; DTS: dense tubular system; GPVI: glycoprotein VI; IP_3: inositol-1,4,5-trisphosphate; PIP_2: phosphatidylinositol-(3,4)-bisphosphate; PIP_3: phosphatidyl-(3,4,5)-trisphosphate; PKC: protein kinase C; PLC: phospholipase C; PS: phosphatidylserine; SFK: Src family kinase; TXA_2: thromboxane A_2; vWF: von Willebrand factor

but PI3Kβ-dependent activation of AKT activation may also require mammalian target of rapamycin (mTOR) complex 2 (Torti et al. 2020).

3.2 Platelet Signalling During Platelet-Leukocyte Interplay

PI3K signalling is not only important for primary haemostasis but also plays an essential role in platelet-leukocyte interactions, which promote leukocyte activation and effector functions and are implicated in the pathogenesis of a broad range of inflammatory and cardiovascular diseases (Schrottmaier et al. 2020). Platelet activation is the prerequisite for the fine-tuning of immune responses, which is mediated either by receptor-mediated cell-to-cell contact or via the release of soluble mediators such as chemokines and cytokines.

Platelet-specific p85α deficiency significantly impairs the ability of platelets to form aggregates with monocytes and neutrophils, thereby decreasing in vivo leukocyte transmigration. This effect is most likely mediated by impaired expression of CD62P and CD40L on the surface of p85α-deficient platelets. Interestingly, neither knockdown nor overexpression of the PI3K antagonist phosphatase and tensin homolog (PTEN) was able to affect platelet-leukocyte interaction (Kral-Pointner et al. 2019). Pharmacological inhibition of PI3Kβ by the specific inhibitor TGX-221 impaired the formation of platelet-neutrophil aggregates following extracorporeal circulation (Jackson 2011). In the same model, platelet-granulocyte aggregate formation, as well as subsequent increase of CD11b on granulocytes, was completely blocked by combined treatment of $P2Y_{12}$ and PI3Kβ inhibitors (Krajewski et al. 2012). Moreover, increased oxidised low-density lipoprotein-induced transmigration of neutrophils in the presence of platelets was reduced by the PI3K inhibitor

LY294002, which also prevented in vitro formation of platelet-neutrophil aggregates (Badrnya et al. 2012). Several other inhibitors suppressing PI3K signalling were shown to reduce agonist-induced formation of platelet-leukocyte aggregates in vitro such as blocking protein kinase casein kinase 2 (Ampofo et al. 2015), and pre-treatment of platelets with the highly selective dual kinase inhibitor VS-5584 that suppressed both PI3K and mTOR activity (Spater et al. 2018). Additionally, in vitro incubation of monocytes with thrombin-activated platelets was shown to increase AKT phosphorylation in monocytes, which was associated with pro-inflammatory calcium signalling in monocytes (Stephen et al. 2013).

4 PI3K Isoforms in Platelets

The PI3K family consists of three different classes, all of which are relevant for platelet function. However, at present class I PI3Ks have been investigated most extensively. Figure 2b shows an overview of the involvement of different PI3K isoforms downstream of key platelet receptors. A detailed summary of the role of PI3K class I isoforms and of class II and class III isoforms for specific platelet functions is given in Tables 1 and 2, respectively.

4.1 Class I

Class I PI3Ks comprise a catalytic p110 subunit that associates with a regulatory subunit, forming heterodimers that are commonly referred to as PI3Kα, PI3Kβ, PI3Kγ or PI3Kδ. Class IA PI3Ks can be further classified into two subgroups. Regulatory subunits of class IA (p110α, p110β, p110δ) associate with one of five regulatory subunits (p85α, p50α, p55α, p85β or p55γ) that enable class IA PI3Ks to recognise and bind to phosphorylated Tyr-X-X-Met motifs of activated receptor tyrosine kinases (RTK), adaptor molecules or to small GTPases (Vanhaesebroeck et al. 2016). In contrast, class IB consists of a single PI3K comprising catalytic p110γ and regulatory p101 or p84/p87 which binds to activated $G_{\beta\gamma}$ proteins to mediate signalling downstream of RTKs and GPCRs (Fougerat et al. 2009; Krugmann et al. 1999).

The predominantly expressed regulatory subunit in platelets is p85α with minor levels of p55 and p50 (Watanabe et al. 2003), whereas platelets express all four catalytic p110 subunits—α, β, γ and δ (Watanabe et al. 2003; Tang and Downes 1997; Zhang et al. 2002; Blair et al. 2014), providing platelets with an assortment of functional PI3K enzymes that mediate degranulation, aggregation and thrombus formation as well as platelet-leukocyte interactions, thus supporting haemostatic and immunological platelet functions. However, their relative contribution to platelet activation and function varies.

4.1.1 Class IA—p110α

PI3Kα plays a subtle, discrete role in platelet signalling and is primarily involved in potentiating and priming of platelet responses to low doses of agonists. Thus, genetic depletion of p110α in mouse platelets affects neither activation of GPIIb/IIIa or α-granule secretion, nor thrombus formation under flow. These observations were similar upon stimulation with different agonists, such as thrombin, a synthetic PAR-4 agonist, collagen-related peptide (CRP) or fucoidan (Blair et al. 2014). In contrast, pre-treatment with the p110α-specific inhibitor PIK-75 results in decreased insulin-like growth factor 1 (IGF-1)-mediated phosphorylation of AKT in murine (Blair et al. 2014) and human platelets (Kim et al. 2007). Similarly, PIK-75 reduces GPVI-mediated AKT phosphorylation, Ca^{2+} mobilisation and dense granule secretion, leading to slightly diminished platelet aggregation.

The effects of PI3Kα inhibition are less pronounced compared to inhibition of PI3Kβ (Kim et al. 2009). PI3Kα inhibition is associated with a reduction of the potentiating effect of IGF-1 on PAR-1-mediated aggregation of human platelets

Table 1 Overview of key platelet functions regulated by class I PI3K

	Class IA			Class IB
	p110α	p110β	p110δ	p110γ
Regulatory subunits	p85α, p85β, p50, p55	p85α, p85β, p50, p55	p85α, p85β, p50, p55	p101, p84/87
Receptor type	TK	TK, GPCR	TK	GPCR
Thrombopoiesis	= Count	= Count	= Count	
Bleeding	= Mouse	↓Mouse, = rat, = rabbit, ↓human	= Mouse, = rat	= Mouse
Thrombosis in vivo	↑Mouse	↑Mouse, = rat, ↑rabbit	= Rat	↑Mouse
Adhesion under flow	↑Coll, ↑vWF, = ADP, = Thr *Priming:* ↑α-PLA, ↓TPO	↑vWF, ↑Coll, ↑Fg *Priming:* ↑α-PLA	= Coll	↑Coll
Aggregation	*Aggregation*: ↑Coll *Priming:* ↑IGF-1, ↑α-PLA, ↓TPO *GPIIb/IIIa activation*: ↑Coll, ↑Thr	*Aggregation*: ↑Coll, ↑Thr, ↑ADP *Priming:* ↑CD40L, ↑α-PLA, ↑TPO *GPIIb/IIIa activation*: ↑Coll *Priming:* ↑TPO ↑clot retraction	*Aggregation*: = Coll	*Aggregation*: = Coll, ↑ADP *GPIIb/IIIa activation*: ↑ADP
Shape/structure	Filopodia (=vWF)	Lammellipodia (↑Fg) Spreading (↑Fg)	Spreading (↑Fg, ↑vWF)	Spreading (↑Fg)

(continued)

Table 1 (continued)

	Class IA			Class IB
	p110α	p110β	p110δ	p110γ
Degranulation	CD62P: ↑Coll, = Thr, = Fuc ATP: ↑Coll Serotonin: ↑Coll *Priming:* ATP: ↓TPO	CD62P: ↑Coll, = Thr, ↑ADP ATP: ↑Coll Serotonin: ↑Coll, = Thr PS: ↑Coll, ↑ADP *Priming:* CD62P: ↑CD40L, ↑TPO	Serotonin: = Coll	Serotonin: = Coll PS: = ADP
Signalling	p-AKT: ↑Coll Ca^{2+}: ↑Coll, = Thr *Priming:* p-AKT: ↑IGF-1, ↑α-PLA	PIP_3: ↑Thr p-AKT: ↑Coll, ↑Thr, ↑Fg Ca^{2+}: ↑Coll, ↑ADP, = Thr, ↑Fg, = vWF Rap1b: ↑Coll, ↑ADP, ↑Fg TXA_2: ↑ADP *Priming:* p-AKT, p-MAPK, Rap1b, TXA_2: ↑TPO	p-AKT: = Coll Ca^{2+}: = Coll, = Thr	p-AKT: = Coll, ↑ADP Ca^{2+}: = Coll, = Thr, = ADP Rap1b, p-MAPK: ↑ADP *Priming:* TXA_2: ↑TPO
Leukocyte interaction		↑PNA (ECC)		↑PLA, ↑neutrophil/monocyte recruitment
Antagonists	*PTEN* p-AKT: ↓Coll, ↓Thr CD62P, Aggregation, PLA: = Coll, = Thr → no effect on function *SHIP* PIP_3, Ca^{2+}, CD62P, ATP: ↓Coll → reduced activation			

PI3K-mediated regulation of platelet responses is depicted via arrows: downregulation (↓), no regulation (=), upregulation (↑). Stimulation by collagen, convulxin or collagen-related peptide is summarised as 'Coll', stimulation by thrombin or synthetic PAR-agonist is summarised as 'Thr'.
ADP/ATP: adenosine di/triphosphate; α-PLA: anti-phospholipid antibody; CD40L: CD40 ligand; ECC: extracorporeal circulation; Fg: fibrinogen; Fuc: fucoidan; GPIIb/IIIa: glycoprotein IIb/IIIa; GPCR: G protein-coupled receptor; IGF-1: insulin-like growth factor 1; p-AKT: phosphorylated protein kinase B/AKT; PAR: protease-activated receptor; PIP_3: phosphatidylinositol-(3,4,5)-trisphosphate; PLA: platelet-leukocyte aggregate; p-MAPK: phosphorylated mitogen-activated protein kinase; PNA: platelet-neutrophil aggregate; PS: phosphatidylserine; Rap1b: Ras-related protein 1b; TK: tyrosine kinase; TPO: thrombopoietin; TXA_2: thromboxane A_2; vWF: von Willebrand factor;

Table 2 Overview of key platelet functions regulated by class II and III PI3K

	Class II		Class III
	PI3KC2α	PI3KC2β	Vps34
Regulatory subunits			Vps15
Thrombopoiesis	*Platelets*: = Count, = size, = lifespan, ↑pro-platelet separation *Megakaryocytes*: = Count, = ploidy, = pro-platelets	*Platelets*: = Count	*Platelets*: ↑Count *Megakaryocytes*: ↑Migration, ↑endocytosis, ↑intracellular trafficking
Bleeding	?↑↓Mouse	= Mouse	= Mouse
Thrombosis in vivo	↑Mouse	= Mouse	↑Mouse
Adhesion under flow	↑Coll, ↑shear	= Coll	↑Coll
Aggregation	*Aggregation*: = Coll, = Thr, = ADP *Fg-binding*: = Coll, = Thr, = ADP	*Aggregation*: = Coll, = Thr, = ADP *Fg-binding*: = Coll, = Thr	?↑↓Aggregation ?↑↓Fg-binding
Shape/structure	*Platelet*: α-granule: ↑count, ↓size ↓OCS size ?↑↓Filopodia *Megakaryocyte*: α-granule: ↑count, ↓size ↓DMS size	*Platelet*: = OCS size	*Platelet*: α-granule: ↑count, ↓size dense granule: ↑count ?↑↓Spreading *Megakaryocyte*: α-granule: ↑count, ↓size
Degranulation	CD62P: = Coll, = Thr, = ADP Serotonin: = Coll, = Thr, = ADP	CD62P: = Coll, = Thr Serotonin: = Thr	?↑↓CD62P ?↑↓ATP ?↑↓Serotonin
Signalling	PI3P: ↑basal, = Coll, = Thr	PI3P: = basal, = Coll, = Thr	PI3P: ↑basal, ↑Coll, ↑Thr

PI3K-mediated regulation of platelet responses is depicted via arrows: downregulation (↓), no regulation (=), upregulation (↑). Unclear regulation with contradictory findings is indicated by question mark (?↑↓). Stimulation by collagen, convulxin or collagen-related peptide is summarised as 'Coll', stimulation by thrombin or synthetic PAR-agonist is summarised as 'Thr'.
ADP/ATP: adenosine di/triphosphate; CRP: collagen-related peptide; ECC: extracorporeal circulation; DMS: demarcation membrane system; Fg: fibrinogen; GPIIb/IIIa: glycoprotein IIb/IIIa; OCS: open canalicular system; PAR: protease-activated receptor; PI3P: phosphatidylinositol-3-monophosphate

(Hers 2007). However, this effect is compensated by p110β in p110α-deficient platelets (Blair et al. 2014). Moreover, the potentiating effect of anti-phospholipid antibodies (α-PLA) on platelet aggregation induced by low doses of thrombin as well as thrombus formation under arterial flow is also mediated by the PI3K isoforms p110α and p110β. Using pharmacological inhibitors and genetic approaches it was

shown that p110α and p110β act downstream of GPIb and Toll-like receptor 2 in a complementary, non-redundant manner (Terrisse et al. 2016).

In contrast to the stimulating role of p110α on IGF-1- and α-PLA-mediated priming, p110α plays an inhibitory role in thrombopoietin (TPO)-mediated priming of platelets: concomitant to increased TPO-induced enhancement of thrombus formation, ATP secretion and CRP-induced platelet aggregation are significantly elevated in p110α-deficient platelets. Interestingly, reduced TPO-mediated platelet priming was independent of p110α kinase activity and was associated with ERK phosphorylation and TXA_2 synthesis (Blair et al. 2018).

Furthermore, GPVI-induced platelet activation and thrombus formation are mildly affected by p110α inhibition (Gilio et al. 2009; Kim et al. 2007), while no effect on bleeding time could be observed even though p110α contributes to thrombus formation in murine in vivo models (Laurent et al. 2018).

Overall, there is a discrepancy between studies using pharmacological inhibition of p110α and genetic knockout of p110α, which can be explained by (1) a degree of functional redundancy between isoforms or compensatory mechanisms (Blair et al. 2014) and (2) targeting of other PI3K isoforms at high inhibitor concentrations.

4.1.2 Class IA—p110β

The critical role of PI3Kβ for pro-thrombotic platelet functions was first recognised in 2005 by Jackson and co-workers (Jackson et al. 2005). Since then it has been identified as the most important PI3K isoform in platelets, being a pivotal regulator of both haemostatic and immunomodulatory functions.

Platelet p110β plays a central and non-redundant role for PI3K signalling. Indeed, platelets lacking p110β show decreased levels of p85α, probably due to p85α instability, which also affects signalling by other isoforms as they lack their main regulatory subunit. Subsequently, p110β deficiency abolishes PI3K kinase activity and $PI(3,4,5)P_3$ production in platelets, leading to reduced phosphorylation of AKT (Martin et al. 2010). Platelet PI3Kβ mediates downstream phosphorylation of AKT at both Ser 473 and Thr 308, resulting in its activation (Torti et al. 2020). In parallel, PI3Kβ also induces PLC-mediated mobilisation of Ca^{2+} from the DTS (Gilio et al. 2009), further demonstrating the interwoven nature and complexity of the intra-platelet signalling network.

Due to its unique ability among platelet PI3Ks to signal downstream of both RTKs and GPCRs, PI3Kβ is induced by a vast variety of physiologic stimuli, including vWF, collagen, thrombin, ADP and fibrinogen. Thus, PI3Kβ regulates both inside-out and outside-in signalling to modulate all stages of primary haemostasis and provides a link to coagulation, as has been shown in a plethora of in vitro experiments using isoform-specific pharmacologic inhibition or platelet-specific knockout mice (Gilio et al. 2009; Martin et al. 2010; Jackson et al. 2005; Laurent et al. 2014).

Circulating platelets are sequestered at sites of endothelial damage by fleeting interactions with vWF via GPIb-V-IX. PI3Kβ favours this unstable interaction to

promote platelet adhesion under flow without affecting GPIb-dependent Ca^{2+} mobilisation. Accordingly, inhibition of PI3Kβ reduces in vitro thrombus formation on vWF-coated surfaces at high shear rates (Jackson et al. 2005; Cosemans et al. 2006). Of note, GPIIb/IIIa-dependent Ca^{2+} flux and feedback via paracrine ADP are required to form stable thrombi and the involvement of PI3Kβ in this will be discussed below (Cosemans et al. 2006; Goncalves et al. 2005).

Platelet adhesion is further stabilised by binding of collagen to GPVI and GPIa/IIa (integrin $\alpha_2\beta_1$) receptors, both of which transduce signals via PI3Kβ. Interestingly, while GPVI signalling was shown to enhance PLCγ activity downstream of PI3Kβ, their respective roles are reversed in GPIa/IIa-mediated signalling where PI3Kβ activity is regulated by upstream PLCγ (Gilio et al. 2009; Consonni et al. 2012).

Studies using pharmacologic inhibition of p110β or genetically modified mice have provided exhaustive evidence of PI3Kβ being an essential mediator of AKT phosphorylation and Ca^{2+} mobilisation induced by collagen or the GPVI-specific agonist convulxin (CVX) (Gilio et al. 2009; Kim et al. 2009). As a consequence, CVX-induced activation of the small GTPase RAP1B, member of RAS oncogene family (Rap1b), which senses changes in cytosolic Ca^{2+} and triggers sustained integrin activation, also depends on PI3Kβ (Canobbio et al. 2009; Stefanini and Bergmeier 2016). Inhibition or deficiency of platelet p110β further abolishes surface expression of CD62P or release of ADP and serotonin from dense granules in response to GPVI stimulation, resulting in diminished platelet aggregation and thrombus formation under flow conditions (Gilio et al. 2009; Kim et al. 2009; Martin et al. 2010; Canobbio et al. 2009). Although GPVI-mediated signalling and subsequent platelet functions such as dense granule release and aggregation are also supported by p110α, its contribution is far outweighed by the impact of p110β (Kim et al. 2009). Similar to GPVI signalling, signal transduction of GPIa/IIa via PI3Kβ also regulates platelet activation by modulating inside-out activation of GPIIb/IIIa and thrombus formation under flow (Consonni et al. 2012).

In addition to regulating these pro-thrombotic functions that aid in the formation of a platelet plug, PI3Kβ also mediates collagen-induced exposure of PS on the platelet surface which thus provides a negatively charged platform for the assembly of coagulation factors (Gilio et al. 2009).

Further linking coagulation with platelet PI3Kβ, thrombin activates PARs that also transduce signals via PI3Kβ to mediate platelet activation and responses. Indeed, inhibition or deficiency of platelet p110β abrogates $PI(3,4,5)P_3$ production and AKT phosphorylation upon thrombin stimulation. Accordingly, platelet aggregation and subsequent clot retraction are also affected by p110β deficiency. However, the contribution of platelet PI3K to thrombin-induced Ca^{2+} mobilisation from the DTS is almost negligible and the importance of PI3K for thrombin-induced aggregation is less pronounced than for collagen (Gilio et al. 2009; Martin et al. 2010). In line with this, CD62P expression and serotonin release upon thrombin stimulation are independent of PI3Kβ and platelet aggregation is only partially impaired by PI3Kβ inhibition (Martin et al. 2010; Jackson et al. 2005). This supports the existence of individual, agonist-specific and only partially overlapping signal cascades within platelets and the distinct role of PI3Kβ therein.

Platelet inside-out activation is propagated by positive feedback mechanisms via ADP and TXA_2. Platelets express two ADP receptors, $P2Y_1$ and $P2Y_{12}$, the former of which is coupled to $G_{\alpha q}$ and primarily signals via PLCβ and downstream SFK activation without involving PI3K (Cosemans et al. 2006). In contrast, $P2Y_{12}$ is coupled to $G_{\alpha i}$ and its downstream effects are partially PI3K-dependent (Kahner et al. 2006). Platelet responses to ADP heavily depend on the class IB isoform PI3Kγ (Hirsch et al. 2001). However, although PI3Kβ was long believed to be solely induced by tyrosine kinases, it has the unique ability among class IA PI3Ks to be also activated by $G_{\beta\gamma}$, and PI3Kβ has since been found to be an important mediator of $P2Y_{12}$ signal transduction and subsequent platelet responses (Kurosu et al. 1997; Garcia et al. 2010).

Indeed, PI3Kβ and PI3Kγ are both required to achieve maximal Rap1b activity and platelet aggregation upon ADP stimulation. Accordingly, inhibition of only PI3Kβ already affects platelet ADP-induced Rap1b activity, CD62P expression and platelet aggregation (Krajewski et al. 2012; Jackson et al. 2005; Cosemans et al. 2006; Canobbio et al. 2009). Of note, while PI3Kβ mediates the generation of the feedback molecule TXA_2, TXA_2-induced Ca^{2+} mobilisation and dense granule release are independent of PI3Kβ. In line with this, PI3Kβ regulates phosphorylation of AKT and ERK as well as platelet aggregation independently of TXA_2 (Jackson et al. 2005; Garcia et al. 2010).

As a secondary feedback molecule, ADP enhances thrombin-induced Ca^{2+} mobilisation and pro-coagulant activity by prolonging PI3Kβ and PLC activation, whereas PI3Kγ does not. This demonstrates the non-redundant roles of PI3Kβ and PI3Kγ for ADP-induced platelet activation (Meijden et al. 2008).

Additionally, other non-classical stimuli may render platelets more sensitive to haemostatic and immunologic stimuli and thus potentiate functional responses. On a molecular level, these priming effects of certain stimuli are due to prolonged or enhanced PI3Kβ signalling. For example, CD40L enhances α-granule secretion, GPIIb/IIIa activation and platelet aggregation in response to GPVI stimulation and in dependency of PI3Kβ signalling (Kuijpers et al. 2015). Similarly, the exacerbating effects of α-PLA on pro-thrombotic platelet functions such as aggregation and thrombus formation under flow can be abolished by PI3Kβ inhibition or genetic deficiency (Terrisse et al. 2016). TPO uses PI3Kβ to increase AKT, MAPK and Rap1b function as well as platelet degranulation, GPIIb/IIIa activation and aggregation upon PAR stimulation (Moore et al. 2019). In contrast, IGF-1 enhances PAR-induced platelet activation primarily via PI3Kα. However, in the absence thereof, IGF-1 effects also require PI3Kβ (Blair et al. 2014).

Eventually, these inside-out signalling events result in activation of GPIIb/IIIa and its binding of fibrinogen. In turn, fibrinogen triggers outside-in signalling which prominently involves PI3Kβ: Engagement of GPIIb/IIIa activates the focal kinase proline-rich tyrosine kinase 2 in dependency of Src-kinases, which subsequently induces PI3Kβ (Cipolla et al. 2013). As a consequence, AKT phosphorylation and Rap1b activity upon adhesion of platelets to fibrinogen as well as GPIIb/IIIa-dependent Ca^{2+} responses under shear are largely abolished by PI3Kβ inhibition which subsequently affects downstream cellular functions (Torti et al. 2020; Jackson

et al. 2005; Cipolla et al. 2013). Genetic abrogation of PI3Kβ activity via kinase-dead forms or platelet-specific deficiency results in reduced platelet adhesion to fibrinogen-coated surfaces both under static conditions and under flow. Furthermore, lamellipodia formation is practically absent in PI3Kβ-deficient platelets (Martin et al. 2010; Canobbio et al. 2009). Interestingly, platelet spreading on fibrinogen appears to require PI3Kβ activity but not AKT phosphorylation (Torti et al. 2020).

In summary, these in vitro studies have provided ample evidence that PI3Kβ regulates both inside-out and outside-in platelet signalling in response to various agonists, although they appear to be differently affected. While collagen responses are heavily dependent on PI3Kβ, thrombin responses only partially rely on PI3Kβ. Thereby, platelet PI3Kβ mediates crucial cellular functions such as platelet activation, adhesion and aggregation, which affects the haemostatic and pro-thrombotic function of platelets. The translatability of these findings and the potential of PI3Kβ as pharmacologic target for anti-thrombotic drug development were evaluated in a number of in vivo settings.

Platelet-specific deletion of p110β in mice prevents stable vessel occlusion following ferric chloride-induced carotid artery injury, providing clean evidence for the cell-specific importance of PI3Kβ for thrombus formation (Martin et al. 2010). These findings are recapitulated by pharmacologic inhibition of PI3Kβ using the isoform-specific inhibitor TGX-221 in thrombosis models in mice, rats and rabbits, revealing greater anti-thrombotic effects than aspirin and underlining the appeal of PI3Kβ as drug target (Jackson et al. 2005; Bird et al. 2011; Sturgeon et al. 2008). Indeed, since their first discovery, PI3Kβ inhibitors have become refined in terms of specificity and solubility while retaining anti-thrombotic efficacy, resulting in the inhibitor AZD6482 among others (Nylander et al. 2012). However, as with all anti-platelet medications, bleeding complications are a considerable concern as targeting platelet function only in times of pathologic thrombosis without affecting their physiologic patrolling function to maintain vascular integrity is an enormous challenge. This puzzle is further complicated by the fact that effects of PI3Kβ on bleeding tendencies appear to be species-specific. While TGX-221 did not increased bleeding time in rats and rabbits (Jackson et al. 2005; Bird et al. 2011; Sturgeon et al. 2008), effects in mice are contradictory as TGX-221 increased rodent tail and renal bleeding times, whereas mice with a kinase-dead form of PI3Kβ showed no alteration of bleeding times (Canobbio et al. 2009; Bird et al. 2011).

Nevertheless, overall, targeting platelet PI3Kβ has proven to yield effective anti-thrombotic effects with limited haemorrhagic complications in preclinical models. Therefore, drugs targeting PI3Kβ are currently in development and under evaluation in clinical studies, which will be discussed later.

4.1.3 Class IA—p110δ

In contrast to the broad expression of p110α and p110β in various different cell types, p110δ is primarily found in haematopoietic cells (Chantry et al. 1997; Vanhaesebroeck et al. 1997) and is known to play an important role in B cell development and

B cell receptor signalling (Clayton et al. 2002; Okkenhaug et al. 2002). Human and mouse platelets show low levels of p110δ expression (Zhang et al. 2002) and mice lacking p110δ or expressing catalytically inactive point-mutated p110δ have normal platelet counts and do not show any overt bleeding disorders (Senis et al. 2005).

Functionally, p110δ seems to play a minor role in downstream signalling of GPVI and is involved in spreading of platelets on fibrinogen and vWF by contributing to outside-in signalling of GPIIb/IIIa (Senis et al. 2005). In agreement with that, spreading defects in p110δ-deficient platelets could be overcome by pre-treatment of platelets with the $P2Y_{12}$ receptor agonist ADP, suggesting that p110δ is not rate-limiting for this process and might play a non-critical role under conditions of strong PI3K activation. Indeed, inhibition of p110δ does not significantly reduce AKT phosphorylation, Ca^{2+} mobilisation, dense granules release, or platelet aggregation upon CVX stimulation (Kim et al. 2009). Similarly, blocking PI3Kδ does not affect thrombus formation on collagen under flow (Cosemans et al. 2006).

4.1.4 Class IB—p110γ

Apart from PI3Kβ, also PI3Kγ plays a significant role in transmitting platelet activation, with its activation and signal transmission being highly specific for ADP-mediated $P2Y_{12}$ stimulation.

Indeed, ADP causes activation of p38 MAPK (Wang et al. 2015) and GPIIb/IIIa and thereby platelet aggregation in a PI3Kγ-dependent manner (Hirsch et al. 2001; Manganaro et al. 1853). However, both PI3Kγ and PI3Kβ are essential for maximal phosphorylation of AKT and activation of the small GTPase Rap1b after stimulation with ADP as well as subsequent platelet aggregation (Canobbio et al. 2009; Woulfe et al. 2002).

In contrast, platelet responses towards collagen via GPVI (Manganaro et al. 1853) and GPIa/IIa (Consonni et al. 2012) as well as towards thrombin are independent of PI3Kγ (Hirsch et al. 2001). In fact, neither inhibition nor genetic deficiency of p110γ affects AKT phosphorylation, Ca^{2+} mobilisation or dense granule release induced by GPVI stimulation (Kim et al. 2009). Although ADP is essential for the positive feedback activation of further platelets, lack of PI3Kγ does not lead to increased bleeding time in murine models (Hirsch et al. 2001).

However, PI3Kγ is crucial for thrombus stability as platelets lacking PI3Kγ do not form stable aggregates when stimulated with low doses of ADP and spread less on fibrinogen (Lian et al. 2005). Moreover, PI3Kγ maintains thrombus integrity under flow in vitro as PI3Kγ deficiency causes detachment of multi-platelet complexes from collagen-coated surfaces. However, formation and maintenance of a thrombus require both PI3Kγ and PI3Kβ. Thus, both isoforms contribute to thrombus stability and act downstream of $P2Y_{12}$, but not of $P2Y_1$ (Cosemans et al. 2006). Murine models of carotid artery injury show that PI3Kγ is also involved in stable thrombi formation in vivo (Lian et al. 2005).

In flow-restricted areas of blood vessels activated platelets enhance intima-media thickening, recruitment of neutrophils and macrophages as well as endothelial activation, which is diminished if platelets lack PI3Kγ (Wang et al. 2015). Inflammatory responses are further influenced by platelet PI3Kγ as ADP-triggered platelet-leukocyte aggregate formation, which supports leukocyte extravasation and activation is also dependent on PI3Kγ (Wang et al. 2015).

4.2 Class II

Class II PI3Ks comprise three isoforms of which two, PI3KC2α and PI3KC2β, are expressed in platelets and megakaryocytes (Mountford et al. 2015; Valet et al. 2015; Petitjean et al. 2016). These kinases catalyse the conversion of phosphatidylinositol (PtdIns) to phosphatidylinositol-3-monophosphate (PI3P) and of phosphatidylinositol-4-monophosphate to phosphatidylinositol-(3,4)-bisphosphate ($PI(3,4)P_2$). While global deletion of PI3KC2β produces viable and fertile mice without overt gross morbidity (Harada et al. 2005), deficiency of PI3KC2α is embryonically lethal (Yoshioka et al. 2012).

4.2.1 PI3KC2α

In order to circumvent the embryonic lethality of global PI3KC2α deficiency, different genetic strategies are employed to study PI3KC2α function, such as heterozygously truncated PI3KC2α (Mountford et al. 2015), kinase-inactive PI3KC2α (Valet et al. 2015) or an inducible PI3KC2α-silencing model (Mountford et al. 2015). While some effects of PI3KC2α deficiency seem clear-cut, other effects still remain somewhat ambiguous as these models yield slightly different results.

Mice lacking functional PI3KC2α contain normal megakaryocyte counts in the bone marrow that also show unaltered ploidy and in vitro pro-platelet formation (Mountford et al. 2015). However, megakaryocytes display altered α-granule content and an enlarged demarcation membrane system which constitutes the membrane reservoir for thrombopoiesis (Mountford et al. 2015; Valet et al. 2015). In line with this, mice with kinase-inactive PI3KC2α have more circulating barbell-shaped pro-platelets (Valet et al. 2015) which may indicate a role of PI3KC2α in platelet production. Nevertheless, lack of functional PI3KC2α does not affect count, size or lifespan of circulating platelets in any of the three murine models (Mountford et al. 2015; Valet et al. 2015).

Similar to megakaryocytes, platelets deficient for PI3KC2α also exhibit structural alterations with a more invaginated cell membrane surface (Valet et al. 2015) that also harbours greater openings into a dilated OCS (Mountford et al. 2015; Selvadurai et al. 2019) as well as altered α-granules (Valet et al. 2015). Interestingly, in contrast to class I PI3K isoforms, PI3KC2α contributes only to production of PI3P under basal conditions of slow turnover, whereas stimulation-induced fast production of

PI3P is not regulated by PI3KC2α (Valet et al. 2015). Neither overall platelet lipid composition nor surface receptor expression is modulated by PI3KC2α (Mountford et al. 2015; Valet et al. 2015; Selvadurai et al. 2019). Yet, platelets with defective PI3KC2α contain reduced levels of membrane skeleton-associated proteins, which points towards a potential role of PI3KC2α in platelet shape change. However, only few studies on PI3KC2α are available yet which also use different genetic models. Consequently, data are still inconclusive about the contribution of PI3KC2α to filopodia formation (Valet et al. 2015; Selvadurai et al. 2019).

In line with the unaltered agonist-induced PI3P levels upon PI3KC2α-deficiency, classic platelet functions such as degranulation, fibrinogen binding, aggregation and in vitro adhesion under static conditions are independent of PI3KC2α (Mountford et al. 2015). However, PI3KC2α regulates shear-dependent platelet adhesion and deposition which influences thrombus stability and embolisation. Accordingly, mice with defective PI3KC2α show delayed thrombus formation in vivo with increased reperfusion events, whereas effects on bleeding time are dependent on the specific model. While abrogation of PI3KC2α expression impacts on haemostasis, kinase inactivation has no effect on bleeding time (Mountford et al. 2015; Valet et al. 2015). Therefore, PI3KC2α has gained interest as a potential drug target to pharmacologically counter thrombotic events without endangering regular haemostasis. Recently, the isoform-specific highly potent inhibitor MIPS-19416 was presented to recapitulate structural alterations of the OCS observed upon PI3KC2α-deficiency and to prevent thrombosis in murine models without impinging on haemostasis (Selvadurai et al. 2020).

4.2.2 PI3KC2β

In contrast to the central contribution of the PI3KC2α isoform to thrombus formation, no overtly essential role of PI3KC2β for platelet function has been discovered yet. Indeed, PI3KC2β-deficient mice have normal platelet counts and show no propensity for increased bleeding or impaired thrombus formation in vivo (Mountford et al. 2015; Petitjean et al. 2016). On a molecular level, platelets lacking PI3KC2β express unaltered levels of surface receptors and show normal α- and dense granule release as well as fibrinogen binding upon activation (Mountford et al. 2015). Concomitant deficiency of both PI3KC2α and PI3KC2β does not exacerbate structural or functional defects beyond those observed upon PI3KC2α silencing, which underlines the non-redundant roles of class II PI3K isoforms in platelets. However, platelets lacking both PI3KC2α and PI3KC2β contain upregulated levels of class III PI3K, which might represent a compensatory mechanism and ensure sufficient PI3P production (Petitjean et al. 2016).

4.3 Class III

Class III PI3K is composed of a single isoform designated vacuolar protein sorting (Vps) 34 which binds to the regulatory subunit Vps15. Vps34 catalyses the phosphorylation of PtdIns to PI3P. Thereby, Vps34 is generally involved in vesicular trafficking, endosome formation and autophagy (Bellio et al. 2020). Vps34 is ubiquitously expressed, including megakaryocytes and platelets, in which it contributes to basal levels and stimulation-induced production of PI3P (Valet et al. 2017; Feng et al. 2014). However, it was only very recently that first studies have started to shed light on the function of Vps34 in megakaryocytes and platelets. In addition to the use of isoform-specific inhibitors, these discoveries were enabled by the generation of two different mouse models of Vps34-deficiency in the megakaryocyte/platelet lineage that target either exon 17 and 18 (Liu et al. 2017) or exon 21 (Valet et al. 2017).

In accordance with the general function of Vsp34 in organelle development, Vsp34 plays an important role in megakaryopoiesis and thrombopoiesis. Megakaryocytes deficient for Vps34 display altered granule biogenesis with reduced numbers but increased size of α-granules as well as defective intracellular trafficking events such as endocytosis and transport into granules. Accordingly, platelets also exhibit reduced α- and dense granule content (Valet et al. 2017). In line with this observation, low levels of PI3P in megakaryocytes are associated with impaired endosome formation and aberrant development of the demarcation membrane system, thus resulting in reduced pro-platelet formation (Bertovic et al. 2020). Furthermore, megakaryocyte chemotaxis is less directionally targeted upon deletion of Vps34 exon 21, which is accompanied by faulty pro-platelet shedding as a higher fraction of released platelets accumulates outside of vessels in the bone marrow. Accordingly, these mice display reduced circulating platelet counts (Valet et al. 2017). Of note, deletion of Vps34 exons 17 and 18 does not affect platelet counts (Liu et al. 2017), pointing towards slight differences between the two mouse models.

Indeed, using genetic and pharmacologic approaches Liu et al. observed decreased platelet activation upon targeting of Vps34 as evidenced by impaired induction of major intracellular signalling hubs, reduced GPIIb/IIIa activation, CD62P expression and reduced dense granule release upon stimulation with low-dose collagen or thrombin. Functionally, this led to decreased platelet aggregation, whereas platelet spreading on fibrinogen was unaltered (Liu et al. 2017). In contrast, Valet et al. observed increased agonist-induced release of dense granule content and decreased spreading on fibrinogen of Vps34-deficient or –inhibited platelets (Valet et al. 2017). However, differences could be influenced by the specific genetic model and by slightly different experimental setups. However, despite these somewhat conflicting observations, studies agree that functional loss of Vps34 reduces platelet adhesion to collagen-coated surfaces under flow and impairs thrombus formation upon ferric chloride ($FeCl_3$)-induced vessel injury without affecting bleeding tendencies (Valet et al. 2017; Liu et al. 2017).

Taken together, it appears very clear that Vps34 plays an important role in megakaryocyte and platelet biogenesis and contributes to pro-thrombotic platelet function; however, the role of PI3K class III in platelets still harbours many secrets that need to be elucidated.

4.4 PI3K Antagonists in Platelets

PI3K activity leads to the generation $PI(3,4,5)P_3$, which is counteracted by three phosphatases: Src-homology 2-containing phosphatase 1 and 2 (SHIP1 and SHIP2) and PTEN, all of which are expressed in platelets (Kral-Pointner et al. 2019; Giuriato et al. 1997; Weng et al. 2010). SHIP1 and SHIP2 are closely related isoenzymes, which cleave the phosphate residue at position 5, leading to the formation of $PI(3,4)P_2$, whereas PTEN removes the phosphate residue at position 3, generating $PI(4,5)P_2$ (Cantley 2002).

4.4.1 PTEN

Although a loss of PTEN in platelets causes an increase in AKT phosphorylation (Kral-Pointner et al. 2019; Weng et al. 2010), which is induced downstream of PI3K, lack of PTEN does not cause hyperreactive platelets as platelet aggregation and degranulation are similar to wild-type platelets (Kral-Pointner et al. 2019). Only at extremely low collagen concentrations, a slight increase in platelet aggregation can be observed (Weng et al. 2010). Also, immunological platelet functions, such as their interaction with innate leukocytes and platelet-supported leukocyte extravasation, are not affected by PTEN loss or overexpression (Kral-Pointner et al. 2019).

4.4.2 SHIP

In contrast to PTEN, SHIP was shown to modulate platelet function after stimulation. Thrombin induces tyrosine phosphorylation of SHIP which correlates with an increase in $PI(3,4)P_2$ and supports relocation of SHIP to the actin cytoskeleton in an aggregation-dependent manner (Giuriato et al. 1997). Platelets lacking both SHIP1 and SHIP2 exhibit elevated cytosolic levels of $PI(3,4,5)P_3$ upon GPVI stimulation, accompanied by an increase in intracellular Ca^{2+} concentrations and CD62P surface expression (Pasquet et al. 2000). Further, in GPVI-activated platelets SHIP1, but not SHIP2, predominantly associates with PKCδ together with Lyn. Subsequent phosphorylation of SHIP1 and PKCδ by Lyn reduces GPVI-triggered dense granule release (Chari et al. 2009), demonstrating that SHIP1 negatively regulates platelet function.

A growing body of evidence suggests that integrin GPIIb/IIIa-mediated outside-in signalling not only enhances platelet function but can also downregulate platelet

activation under specific circumstances. This could be at least partially mediated by the activation of SHIP1, which terminates PI3K/AKT signalling and thereby platelet activation. Moreover, SHIP1 inhibitors prevent platelet disaggregation and enhance platelet ATP release (Dai et al. 2016).

Thus, SHIP and not PTEN is predominant in terminating PI3K signalling in platelets to enhance disaggregation.

5 PI3K Inhibitors and Platelets in Clinical Practice

Due to their minor role in platelet function, lack of functional PI3Kα or PI3Kδ is unlikely to affect haemostasis. Furthermore, deficiency of PI3Kγ does not result in exacerbated bleeding tendency despite its central role in maintaining ADP-mediated positive feedback for platelet activation (Hirsch et al. 2001). Therefore, targeting any of these three isoforms in a clinical setting is likely to show only little efficacy in preventing thrombotic events. Further thrombotic complications as a side effect of PI3Kα, PI3Kγ or PI3Kδ inhibition in other pathologic settings are also unlikely. Considering the broad expression profile of p110α and the predominant roles of p110γ and p110δ for immune cells, their systemic inhibition would result in defective immune response.

In contrast, PI3Kβ is a central signalling enzyme in platelets and both haemostatic and pro-thrombotic functions rely on its functionality. Stable incorporation of new platelets into thrombi under high shear stress is dependent on intact PI3Kβ activity. Therefore, while mice with platelet-specific deficiency of p110β are resistant to $FeCl_3$-induced arterial thrombosis, they are not protected from thromboembolisms due to the instability of formed thrombi (Martin et al. 2010; Laurent et al. 2015). Nevertheless, PI3Kβ has raised considerable interest as a potential drug target to counteract thrombotic events.

Initial studies on the anti-thrombotic effects of platelet PI3Kβ inhibition were performed with TGX-221, which was widely used in preclinical settings despite some drawbacks such as solubility that rendered it poorly suitable for in vivo application in humans. Continuous refinement led to the development of structural analogues with more favourable chemical properties while retaining isoform-specificity and efficacy. Among these analogues, AZD6482 has entered clinical trials (Nylander et al. 2012).

In a randomised, double-blind, placebo-controlled, single-dose escalation study AZD6482 has proven to be well tolerated by healthy volunteers and demonstrated efficient ex vivo platelet inhibition with significant but minor prolongation of bleeding time (Nylander et al. 2012). When comparing AZD6482 to current anti-platelet therapy, concomitant application of aspirin and AZD6482 showed stronger platelet inhibition than current dual anti-platelet therapy comprising of aspirin and the $P2Y_{12}$ antagonist clopidogrel. At the same time, combined treatment with aspirin and AZD6482 prolonged bleeding time of healthy volunteers 3.6-fold more than aspirin alone, whereas current dual anti-platelet therapy raised bleeding time the most with values 8.1-fold over aspirin (Nylander et al. 2015).

Of note, despite the ubiquitous expression of p110β, AZD6482 only led to minor changes in glucose homeostasis upon supra-therapeutic dosage that was attributed to unspecific inhibition of PI3Kα. Thus, PI3Kβ inhibition is believed to bear no clinically relevant metabolic risk when used short-term at therapeutic dosage as acute anti-thrombotic treatment (Nylander et al. 2012).

Currently, no large-scale trial investigating PI3K inhibitors on thrombotic events is under way. However, AZD6482 has also proven efficient at blocking progression of PI3Kβ-driven and PTEN-deficient tumours in murine xenograft models (Ni et al. 2012) and PI3Kβ inhibitors are currently under investigation in interventional clinical phase I trials against metastatic tumours with PTEN deficiency (NCT03131908) or mutations of p110β or PTEN (NCT03218826) (National Cancer Institute 2018; M.D. Anderson Cancer Center 2017).

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PI3K and AKT Isoforms in Immune-Mediated Diseases

PI3K Isoforms in Vascular Biology, A Focus on the Vascular System-Immune Response Connection

Daniela Carnevale, Giuseppe Lembo, and Sara Perrotta

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Abstract Cardiovascular diseases are the most common cause of death around the world. Hypertension and atherosclerosis, along with their sequalae and consequent target organ damage, constitute the main vascular risk factors contributing to the onset of cardiovascular disease. Disturbances in the homeostatic relationship established among the various components of the vascular milieu—namely endothelial and smooth muscle cells, adventitia, immune cells, and fibers of the autonomic nervous system—trigger the development of these arterial pathologies. In terms of molecular targets involved in vascular dysfunction and appealing for therapeutic purposes, the multitude of functions that phosphoinositide-3-kinases (PI3K) perform has become an attractive area of investigation in the field of arterial diseases. Composed of eight members arranged in III different classes based on their structure and substrate specificity, PI3Ks are characterized by their shared capability to produce phosphoinositides but, at the same time, they provide specificity and non-redundancy, owing to differences in expression levels of each member in different cell components of the vascular environment, different activation mechanisms and specific subcellular locations. This chapter aims at providing an overview of the functions of the different

D. Carnevale (✉) · G. Lembo · S. Perrotta
Department of Molecular Medicine, "Sapienza" University of Rome, 00161 Rome, Italy
e-mail: daniela.carnevale@neuromed.it; daniela.carnevale@uniroma1.it

D. Carnevale · G. Lembo
Department of Angiocardioneurology and Translational Medicine, "IRCCS Neuromed", 86077 Pozzilli (IS), Italy

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PI3K isoforms identified thus far in the vasculature, focusing on the emerging relationship established by components of the vascular and immune systems, at the steady-state and during pathology.

Abbreviations

Ang II	Angiotensin II
ALLN	N-acetyl-leucyl-leucyl-norleucinal
ECs	Endothelial cells
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GEF	GDP/GTP exchange factors
hcECs	Human coronary endothelial cells
HEK293 cells	Human embryonic kidney 293 cell line
HUVEC	Human umbilical vein endothelial cells
IGFR	Insulin growth factor receptor
LTCC	L-type voltage-gated calcium channel
MLC	Myosin light chain
MLCP	Myosin light chain phosphatase
NO	Nitric oxide
NOS	Nitric oxide synthase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphoinositide-3-kinase
PLC	Phospholipase C
RBL-2H3 cells	Basophilic leukemia cell line isolated from rat
RTK	Tyrosine-kinase receptor
S1P	Sphingosine-1-phosphate
SMCs	Smooth muscle cells
TCR	T cell receptor
TRPC6	Transient receptor and potential canonical channel C6
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

A considerable amount of work suggests that the signaling mediated by PI3Ks (Table 1) is responsible for numerous and diverse homeostatic and pathological functions in the cardiovascular and immune systems. Signals downstream of G protein-coupled receptors (GPCRs) are the main activators of the PI3K pathway in immune cells and in cells of the cardiovascular system, as well as those involved in the regulation of vascular function. Once activated, GPCRs determine the dissociation of the G protein α subunit (Gα) from the Gβγ dimer, which in turn binds the regulatory subunit and activates the class IB catalytic subunit p110γ, which has been described as a regulator of leukocytes, especially for processes mediating their recruitment to inflammatory sites (Costa et al. 2011; Rommel 2010; Hirsch et al. 2000), and of cells belonging to the cardiovascular system (Carnevale and Lembo 2012; Ghigo et al. 2017).

Compelling studies unraveled that an underlying mechanism common to both cardiovascular and immune systems is related to the role played by PI3Ks in the regulation of intracellular AKT and Calcium (Ca^{2+}) pathways. In this chapter, we will highlight relevant works investigating the role of PI3Ks at the crossroads of vascular and immune functions (Table 1). Future works will be necessary to clarify how these systems crosstalk in health and disease and how PI3Ks contribute to this relationship.

2 PI3Ks and Vascular Biology

Many cell components participate in the regulation of vascular function in health and disease. Endothelial cells, vascular smooth muscle cells (SMCs), and adventitial tissues continuously interplay with fibers of the autonomic nervous system, with inflammatory and immune cells and vasa vasorum to keep homeostatic conditions that are necessary for the various functions carried out by the vascular system. The interactions among these cells/tissues and the secretion of growth factors, cytokines/chemokines, and extracellular matrix components, overall allow vascular homeostasis. When internal or external stimuli perturb this homeostatic system, vascular dysfunction ensues, contributing to cardiovascular diseases such as atherosclerosis and hypertension. While the role of growth factors secreted from vascular SMCs and participating in vascular remodeling, the contribution of oxidative stress in deteriorating endothelial function and the potent effects induced by autonomic imbalance on vascular homeostasis are all well-known determinants of vascular dysfunction (Hill et al. 2006; Salabei et al. 2013; Lupieri et al. 2020; Kaplan-Albuquerque et al. 2003; Esler 2015), only more recent studies have shed light on the crucial role played by immune and inflammatory mechanisms in these processes.

One of the key intracellular pathways regulating vascular function is represented by Ca^{2+} fluxes. Both endothelial and SMCs functions are profoundly affected by variations in Ca^{2+} fluxes at the steady-state and during pathological variations. In all

Table 1 Summary of PI3Ks vascular and immune functions involved in vascular biology

Vascular disease	Experimental or clinical condition	Species	Cell type	PI3K isoform (s)	Activity	References
Vascular dysfunction	Administration of S1P, bradykinin, and VEGF	Bovine aortic endothelial cells	ECs	Class IA PI3Kα and β	Modulation of vascular tone	Igarashi and Michel (2001)
	Administration of growth factors	Human and bovine cells	ECs	Class I PI3K/Akt	Angiogenesis	Simons et al. (2016), Su et al. (2006), Youn et al. (2009), Saido et al. (1992)
	Inhibition of PI3Kα activity	Mouse	ECs	Class IA PI3Kα	Permeability	Cain et al. (2010), Graupera et al. (2008), Vantler et al. (2015)
Aortic aneurysm	Chronic peripheral AngII infusion	Mouse	ECs	Class II PI3K-C2α	Permeability	Yoshioka et al. 2012; Biswas et al. (2013), Aki et al. (2015)
Hypertension	Inhibition of PI3K activity	Mouse	ECs	Class I PI3Kα, δ and γ	Controlling inflammatory cell recruitment and trafficking	Puri et al. (2004), Puri et al. (2005), Cain et al. (2010)
	Chronic peripheral AngII infusion	Mouse	SMCs	Class IB PI3Kγ	Modulation of L-type calcium channels and vascular tone	Carnevale et al. (2012), Vecchione et al. (2005), Monet et al. (2012)
	Inhibition of PI3K activity	Mouse	SMCs	Class II PI3K-C2α	Smooth muscle cell contraction	Azam et al. (2007), Wang et al. (2006), Yoshioka et al. (2007)

(continued)

Table 1 (continued)

Vascular disease	Experimental or clinical condition	Species	Cell type	PI3K isoform (s)	Activity	References
	Chronic peripheral AngII infusion	Mouse	T cells	Class IB PI3Kγ	T cells activation, migration, and differentiation	Carnevale et al. (2012)
Atherosclerosis	Selective smooth muscle cell inactivation of PI3K isoforms	Mouse	SMCs	Class IA PI3Kα, β and δ	Proliferating/ migratory phenotype	Kaplan-Albuquerque et al. (2003), Vantler et al. (2015)
		Mouse		Class IB PI3Kγ	Migratory phenotype	Fougerat et al. (2008)
	Modulation of autophagy process	Mouse		Class III PI3K	Cells survival	Salabei et al. (2013), Zheng et al. (2012)
	Hypercholesterolemic diet or mechanical arterial injury	Mouse	Mac	Class IB PI3Kγ	Foam cell formation	Chang et al. (2007)
		Mouse	T cells		Intimal hyperplasia	Fougerat et al. (2008), Smirnova et al. (2014)
	Genetic inhibition of PI3Kδ	Mouse	B cells	Class IA PI3Kδ	Differentiation and survival of B cells and atheroprotective function	Okkenhaug et al. (2002)

ECs endothelial cells; *SMCs* smooth muscle cells; *Mac* macrophages

contractile cells, the physiological regulation of Ca^{2+} is maintained through an array of kinases that, by phosphorylating Ca^{2+} channels or adaptor proteins, finely control the opening/closure cycle state. Interestingly, an increasing body of works highlights a crucial role of the PI3K/AKT signaling in the homeostatic regulation of Ca^{2+} fluxes in the cardiovascular system (Macrez et al. 2001; Quignard et al. 2001; Vecchione et al. 2005; Ghigo et al. 2017), with compelling evidence revealing molecular mechanisms that in endothelial cells or in SMCs, underlie important pathophysiological implications. Moreover, the growing number of studies showing that immune cells participate in the vascular milieu both at the steady-state and in diseases, open a door to the investigation of additional roles of the PI3K/AKT signaling which is, in addition, well known as a crucial intracellular pathway in immune cells.

Pathological conditions affecting the vasculature, such as hypertension and atherosclerosis, are characterized by a profound remodeling of the vasculature, a process that is becoming increasingly known to constantly interact with components of the immune system. Taken together, the above considerations suggest that PI3Ks signaling may be a common mediator of the several components, which in various ways, participate in the maintenance of steady-state homeostasis or in the onset of pathological alterations ensuing in the vasculature.

2.1 PI3Ks and Endothelial Cell Functions

Arteries are lined by endothelial cells, which provide gatekeeper functions at the blood/tissue barrier, allowing a controlled transport of nutrients and oxygen, and the recruitment of circulating cells when necessary. Also, the endothelial layer contributes to maintaining the vascular tone by releasing vasoactive molecules like nitric oxide (NO), synthesized by the endothelial NO synthase (eNOS).

PI3Ks are involved in a multitude of physiological and pathological roles of the endothelium (Fig. 1). The use of pan-inhibitors of PI3Ks evidenced a crucial role of PI3K signaling in eNOS activation and consequent NO synthesis in various conditions, including shear stress (Dimmeler et al. 1999), in response to bradykinin (Harris et al. 2001) and to estrogens (Haynes et al. 2000). Other works also clarified more specific roles of selective PI3Ks isoforms, finding that class IA PI3Kα and β are implicated in vascular endothelial growth factor (VEGF)-induced eNOS activation, and PI3Kβ in sphingosine-1-phosphate (S1P) induced eNOS phosphorylation (Igarashi and Michel 2001).

One endothelial-dependent function profoundly affected by PI3Ks signaling is angiogenesis, a process deriving from a combination of endothelial cell migration and proliferation. When molecules such as vascular endothelial growth factor (VEGF) and other growth factors, including epidermal growth factor (EGF) or fibroblast growth factor (FGF), are released in the vascular milieu, intracellular signaling molecules involving PI3Ks and Ca^{2+} signaling are recruited (Simons et al. 2016). Once activated, the cognate receptors induce tyrosine phosphorylation of the intracellular domain, a process responsible for recruitment of phospholipase C (PLC) and

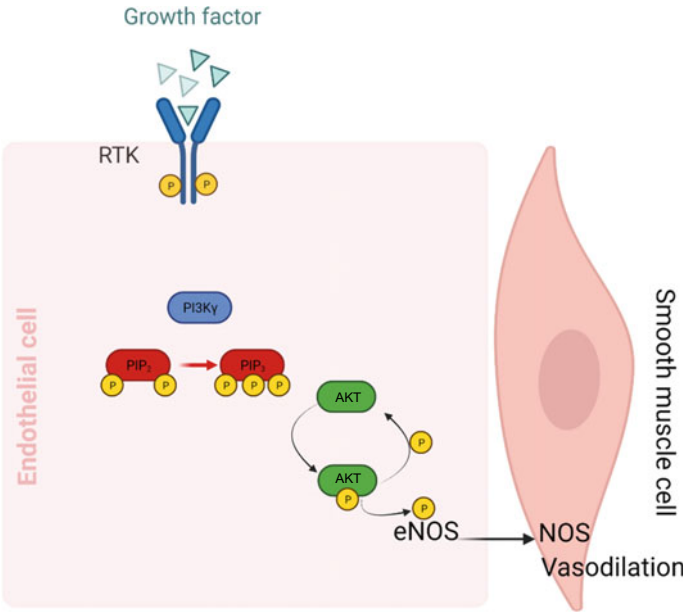

Fig. 1 PI3Ks are involved in important signaling of the endothelium. An example of the crucial role of PI3Kγ signaling in the activation of eNOS and consequent synthesis of NO, causing vasodilation in smooth muscle cells. Created by BioRender.com

activation of PI3Ks. In turn, PI3K/AKT phosphorylation promotes the downstream phosphorylation of eNOS in a Ca^{2+}-dependent way, lastly profoundly affecting angiogenic processes (Simons et al. 2016; Nakagawa et al. 2013).

A series of studies highlighted the existence of a relationship between modulation of Ca^{2+} fluxes and PI3K/AKT signaling, which might contribute to homeostatic and angiogenic processes in endothelial cells. Reorganization and migration of endothelial cells are controlled at the level of cytoskeleton modifications, a process tightly regulated by calpain proteins, through the association with the specific cytoskeletal substrates (Zhang et al. 2017). Calpain proteins are cytosolic cysteine proteases that, once activated, translocate to the plasma membrane to cleave their substrates. In this regard, it has been shown that calpain 2 is involved in VEGF-mediated PI3K/AKT signaling, leading to NOS phosphorylation and NO production (Su et al. 2006). In vitro experiments with bovine aortic endothelial cells, have demonstrated that VEGF induced a marked increase in endothelial cell production of NO, detected by electron spin resonance. Hence, the pharmacological inhibition of calpain 2 with N-acetyl-leucyl-leucyl-norleucinal (ALLN) or Calpeptin was effective in preventing the VEGF-mediated NOS activation and NO production (Youn et al. 2009). An interesting parallel observation also demonstrated that calpain proteins can be activated by interaction with phosphoinositides at the plasma membrane (Saido et al. 1992).

Therefore, it is conceivable that a complex comprising PI3K, membrane phosphoinositides, and calpain is formed at the plasma membrane where it is able to promote angiogenic processes in endothelial cells.

Additional functions of PI3Ks have to do with the structural properties of the endothelial cell barrier. It is well known that the prolonged or continuous exposure to stress or injury may induce loss of integrity of the endothelial compartment (Gunduz et al. 2019; Smirnova et al. 2014). In this context, the process of re-endothelization following mechanical injury in arteries has been proposed as a potential protective mechanism against formation of neointimal layers. All these processes are strictly dependent on migration and proliferation of endothelial cells and have been found to be profoundly influenced or regulated by PI3Ks signaling (Smirnova et al. 2014).

Both PI3Kβ and γ isoforms have been found to be involved in in vitro migratory processes induced by S1P, by using the selective inhibitors of PI3Kβ or γ isoforms. To better investigate the downstream mechanism, Heller et al. used a specific siRNA treatment to induce the downregulation of PI3Kβ or γ expression in human umbilical vein endothelial cells (HUVEC). They showed that only PI3Kβ can induce the migration of endothelial cells by S1P-induced AKT phosphorylation (Heller et al. 2008). Another work found that the depletion of Class IA PI3K in HUVEC cells transfected with siRNA in order to target the p110α isoform increased the cell–cell adherent junctions (measured as junctional index or the number of cells in the junctional area) and reduced the endothelial permeability induced by in vitro TNF stimulation (Cain et al. 2010). The effect on endothelial cell permeability mediated by class IA PI3Kα was ascribed to the formation of Rac1-dependent vascular endothelial cadherin (VE-cadherin) complexes (Cain et al. 2010). Interestingly, another study observed that class I PI3Kα plays a crucial role downstream of the VEGF receptor (VEGFR) through the activation of another small GTPase—RhoA (Graupera et al. 2008). In an in vitro experimental model of cell migration, the authors showed that genetic or pharmacological inactivation of PI3Kα reduced the activity of RhoA with a consequent inhibition of cell migration. These data suggest a differential ability of PI3Kα to recruit different intracellular mediators, depending on the receptor engaging PI3Kα, Since the conclusions of this work proposed that these PI3Kα–dependent mechanisms could mediate reparative processes following arterial injury, a subsequent work aimed at pharmacologically targeting PI3Kα activity observed a negative effect on endothelial cell proliferation induced by VEGF. The authors assessed the effects of PI3Kα inhibition by PIK-75, an isoform-specific small molecule inhibitor, in human coronary SMCs and in human coronary endothelial cells (hcECs). They showed that p110α inhibition also inhibited PDGF and VEGF-induced proliferation and chemotaxis of these cells in a concentration-dependent manner (Vantler et al. 2015) (Table 1). However, contrasting results were obtained in another study which reported similar effects induced by PI3Kα inhibition on thrombosis and neointima formation but not on re-endothelization and proliferation processes (Holy et al. 2014), thus strongly indicating the necessity of further works that carefully examine cell-specific and therapeutically relevant roles of PI3Kα inhibition in this context. In this regard, some years later additional evidence reported a role for class II PI3Ks in controlling permeability of endothelial cells (Yoshioka et al. 2012), highlighting an

additional level of complexity in terms of specific roles of various PI3Ks isoforms. In an in vitro model of PI3K-C2α-depleted endothelial cells, it has been demonstrated that the specific deficiency of this isoform resulted in a defective delivery of VE-cadherin to endothelial cell junction (Yoshioka et al. 2012). Moreover, the PI3K-C2α isoform is involved in S1P-induced cell migration via a Rac1-mediated process (Biswas et al. 2013) and on TGF-β1-induced endothelial cell migration (Aki et al. 2015).

Other relevant functions of the PI3K family in pathological conditions of the endothelial compartment are related to processes controlling inflammatory cell recruitment and trafficking (Yoshioka et al.) (Table 1). Notably, it has been shown that the PI3Kγ isoform is involved in E-selectin-mediated neutrophil recruitment. Indeed, the ablation of PI3Kγ in microvessels significantly reduced the attachment and enhanced rolling of wild-type neutrophils observed in response to TNF stimulation (Puri et al. 2005), suggesting an involvement of class IB PI3K in the vascular-immune crosstalk at the endothelial level. A similar defect in neutrophil adhesion was found in mice treated with a selective inhibitor of the δ-isoform of class IA PI3Ks and confirmed in in vitro inhibition of δ isoform in endothelial cells (Puri et al. 2004). These evidences implied that α, δ and γ isoforms of class I PI3Ks are all involved in efficient neutrophil adhesion and rolling on the endothelium.

Interestingly, while PI3Kα has been classically considered as the isoform activated by RTKs, like VEGF receptor, a report proposed that PI3Kγ may convey VEGF-mediated signaling, affecting endothelial stimulation, through a pathway dependent on H-Ras (Serban et al. 2008). Whether the interaction between RTKs and PI3Kγ is established directly or through indirect mediators depending on GPCR agonist, is yet to be established. The role of PI3Ks in angiogenesis and vascular anomalies related to vascular tumors and malformations has been extensively reviewed elsewhere (Castillo et al. 2016; Kobialka and Graupera 2019).

2.2 PI3Ks and Smooth Muscle Cell Functions

The manifold roles of PI3Ks in vascular biology are also reflected by their crucial involvement in the homeostatic regulation of SMCs functions, as well as in the development of pathological alterations. SMCs carry out several functions in the vascular system, with a variety of specializations depending on the vascular district. A typical function of SMCs at the steady-state is represented by the capability to maintain and regulate vascular tone through their contractile potential. Vascular contraction in resistance arteries is mediated by two main functions of the smooth muscle: one dependent on the effect of agonist-mediated contraction and a second one related to the myogenic tone (Fig. 2), defined as the physiological capability to counteract intraluminal perfusion pressure (Carnevale and Lembo 2012; Perrotta et al. 2016; Kauffenstein et al. 2012). In particular, the myogenic response is generated as an intrinsic contraction of SMCs in response to a variety of stimuli that require a tight regulation of tissue perfusion (Meininger and Trzeciakowski 1988; Hill et al. 2006;

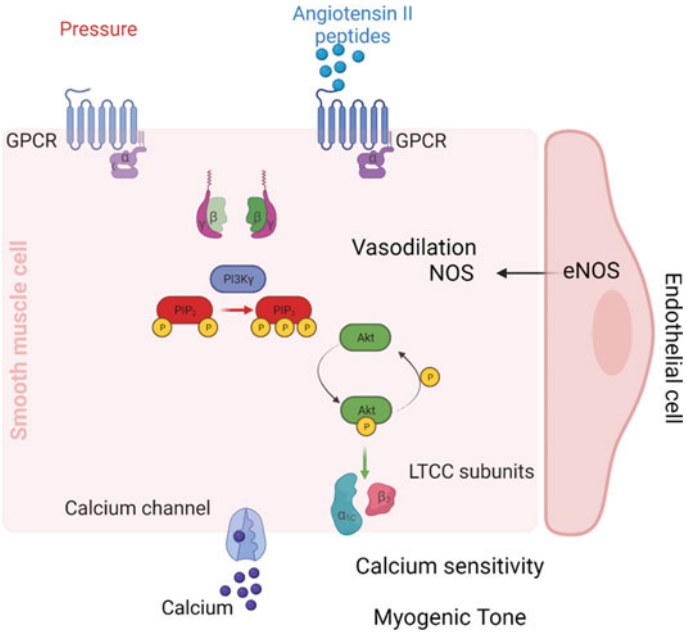

Fig. 2 PI3Ks are involved in the homeostatic regulation of smooth muscle cell function. Vascular contractility is mediated by increased intracellular calcium influx through the L-type voltage-gated calcium channel (LTCC) in turn activated by PI3Kγ signaling in smooth muscle cells, recruited transmembrane GPCRs. Conversely, vascular relaxation is activated by NO synthesis in smooth muscle cells. Created by BioRender.com

Carnevale and Lembo 2012). While this process has been recognized as a protective mechanism from excessive blood flow in a tissue, at the same time longstanding conditions of increased myogenic tone might contribute to an increase in peripheral vascular resistance, hence contributing to the maintenance of chronic hypertensive status and consequent target organ damage (Carnevale and Lembo 2012).

The process of myogenic contraction is generally described as organized in three different phases: (i) the first phase—that is also called basal tone—is characterized by an increased intracellular calcium influx through the L-type voltage-gated calcium channel (LTCC); (ii) the second phase is represented by a further constriction of the SMCs apparatus, in the absence of additional variations of intracellular Ca^{2+}; (iii) in the third and last phase, smooth muscle loses the capability to counteract the mounting perfusion pressure and a spontaneous adaptive dilation of the arterial wall occurs (Carnevale and Lembo 2012; Hill et al. 2006). All the PI3K isoforms have been identified in SMCs, but among them, the γ-isoform could be considered the most relevant isoform for the regulation of vascular contraction, since it has been shown that it regulates Ca^{2+} flux in SMCs. (Carnevale and Lembo 2012).

An initial observation reporting a crucial role of class IB PI3Kγ in the contractile function of SMCs was reported in 2001 when correct functioning of L-type voltage-gated calcium channel (LTCC) was found to be dependent on PI3Kγ (Quignard et al. 2001; Macrez et al. 2001). Given the crucial role of the LTCC-dependent mechanism in regulating vascular contraction, hence contributing to blood pressure regulation, the role of PI3Kγ started to be investigated in the setting of experimental hypertension (Carnevale and Lembo 2012) (Table 1). Mice specifically lacking the γ-isoform of the PI3Ks were instrumental for the advancement of knowledge in this field (Hirsch et al. 2000).

PI3Kγ$^{-/-}$ mice are protected from hypertension induced by the chronic infusion of the peptide hormone Angiotensin II (AngII) (Vecchione et al. 2005; Carnevale et al. 2012). Moreover, mice expressing an inactive catalytic form of PI3Kγ (PI3Kγ kinase-defective KD mice, PI3Kγ $^{KD/KD}$ mice) show a similar protection from AngII-induced hypertension (Carnevale et al. 2012), indicating that the mechanism involved in the phenotype counteracting blood pressure increase is dependent on the kinase activity of PI3Kγ and not on other reported docking functions of PI3Kγ (Carnevale and Lembo 2012; Perino et al. 2011). Another work showed that the direct injection of the phosphoinositide PIP3 into vascular SMCs reproduces the effects of AngII on LTCC, further supporting the notion that the lipid kinase activity of PI3Kγ is crucial for this process (Le Blanc et al. 2004). Interestingly, a similar mechanism has been described in cardiomyocytes, whereby another PI3K isoform—PI3Kα—regulates the LTCC function through an AKT-mediated signaling cascade that lastly phosphorylates a specific subunit of the LTCC channel (the CaVβ2a), protecting the pore-forming subunits from degradation and hence enhancing Ca^{2+} entry (Reinartz et al. 2014). Experiments conducted with a selective PI3Kγ inhibitor or with the PI3Kγ$^{-/-}$ murine model demonstrated that PI3K-mediated AKT phosphorylation is crucially important in SMCs to regulate the probability that the LTCC channel is in the open state by a mechanism similar to the one described in cardiomyocytes (Carnevale et al. 2012). An additional role for PI3Ks in regulating SMCs Ca^{2+}-dependent mechanisms has been described in relation to the activation of the transient receptor and potential canonical channel C6 (TRPC6). TRPC6 is a cation channel that translocates to the plasma membrane upon stimulation of Gq-protein-coupled or tyrosine-kinase receptor. It has been described that the inhibition of PI3K by PIK-93 and wortmannin decreases the translocation of TRPC6 to the plasma membrane and Ca^{2+} entry into HEK293 cells (Human embryonic 293 cell line) (Monet et al. 2012).

In contrast to class IB, the role of class II PI3K has been less clearly defined. A study reported that PI3K-C2α is involved in SMCs contraction. It has been demonstrated that PI3K-C2α is required for Ca^{2+}-induced Rho- and Rho kinase-dependent negative regulation of myosin light chain phosphatase (MLCP) and consequently myosin light chain (MLC) phosphorylation, resulting in SMCs contraction. In particular, in vitro studies with aortic rings showed that KCl membrane depolarization induces stimulation of Ca^{2+} influx across the plasma membrane through the voltage-dependent Ca^{2+} channels. The Ca^{2+} influx induced PI3K-C2α stimulation, which led to Rho activation resulting in MLCP inhibition that promoted SMCs contraction. (Azam et al. 2007; Wang et al. 2006; Yoshioka et al. 2007) (Table 1).

When pathological conditions perturb vascular homeostasis, SMCs are also able to switch their main contractile phenotype toward a synthetic response, characterized by cell proliferation and enhanced migratory properties. In general, the shift of vascular SMCs from a purely contractile apparatus toward a proliferating/migratory phenotype is stimulated by an inflammatory stressful event. This switch is typically observed during the progression and evolution of atherosclerosis. A number of different receptors are involved in the process of proliferation of SMCs in response to growth factors: platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and insulin growth factor receptor (IGFR). A common trait of all these receptors is their ability to recruit common signaling pathways, which includes PI3K, thus providing appealing molecular mechanisms that could be targeted by novel pharmacological drugs. Also, the wide vascular expression of class I PI3Ks has stimulated intense research on the role played by class I PI3K signaling in vascular homeostasis and during pathology. The PDGF/PDGFR pathway has been considered the prototype mechanism regulating smooth muscle cell proliferation and migration and its ability to recruit PI3K signaling was observed many decades ago (Owens et al. 2004; Kaplan et al. 1987). The RTK signaling recruited by PDGF/PDGFR in SMCs determines a sustained activation of PI3Kα but not PI3Kβ (Kaplan-Albuquerque et al. 2003). Interestingly, it was also observed a time-delayed activation of the class IB PI3Kγ dependent on the autocrine/paracrine secretion of GPCR agonists (Yao et al. 2014). At the functional level, while PI3Kα was involved in smooth muscle cell proliferation and migration, experiments conducted on mouse aortic SMCs treated with a selective PI3Kγ inhibitor or with aortic SMCs from PI3K$\gamma^{-/-}$ or PI3K$\gamma^{KD/KD}$ murine models demonstrated that the γ-isoform was involved only in the migratory process (Fougerat et al. 2012).

Building on these results, a series of studies have been conducted to investigate the possibility of inhibiting various class IA isoforms with the aim of preventing SMCs proliferation and migration, a process particularly relevant in the clinical contexts where neointima formation and restenosis are involved (Cain et al. 2010; Graupera et al. 2008; Vantler et al. 2015) (Table 1). By exploiting a strategy of gene silencing, it was shown that PI3Kα has an essential role in SMCs proliferation in response to PDGF and that, unexpectedly, silencing of p110 PI3Kδ affects SMCs proliferation as well (Vantler et al. 2015). In order to find mechanistic evidence for the involvement of p110 PI3Kα in the restenosis process, a mouse model with a selective smooth muscle cell inactivation of PI3Kα was generated and subjected to balloon injury, showing significant protection against neointima formation (Vantler et al. 2015). While this work clarified the role played by class IA PI3Kα, the data obtained on the δ-isoform were less definitive. In fact, ablation of PI3Kδ in SMCs was not protective in the balloon injury-induced damage and neointima formation. However, interestingly, non-catalytic functions for PI3Kδ were demonstrated, suggesting the possibility that this isoform could play additional docking functions in a similar manner to that observed for PI3Kγ (Vantler et al. 2015). Regarding the role of PI3Kβ, only in vitro

data have shown a role in modulating SMCs proliferation and neointima formation. However, the lack of definitive in vivo data hampers from drawing definitive conclusions on its relevance as a potential therapeutic option.

Similar to what is observed for PI3K-dependent mechanisms involved in the regulation of the endothelial compartment and in SMCs contraction, the role of class II and III PI3Ks in arterial disease has been less investigated with few exceptions. Several years ago it was shown a possible role of class II PI3Ks in SMCs migration (Paulhe et al. 2002) and, more recently, the class III PI3K was involved in autophagy (Salabei and Hill 2013; Zheng et al. 2012). In fact, while basal levels of autophagy are fundamental for maintaining adequate vascular functions, it is becoming increasingly clear that a dysfunction of autophagy may contribute to arterial diseases such as atherosclerotic processes (Jia et al. 2006). Interestingly, the initiation of autophagy is regulated by VPS34, which assembles with proteins that are relevant for the core autophagy-related complexes, including Beclin, Ulk1, and others. In this regard, several stimuli such as reactive oxygen species derived from oxidative stress, inflammatory signaling, mitogens, metabolic stressors, and the alteration of pathways regulating cellular homeostasis proved effective in inducing autophagy in SMCs (Salabei and Hill 2013; Zheng et al. 2012), implicating class III PI3K in SMCs survival. Additional autophagy-dependent functions have been involved in the balance between contractile and synthetic functions of SMCs, supporting the hypothesis that the transition from contractile to synthetic function occurs when autophagy is induced to remove contractile proteins and to allow the synthetic/proliferative phenotype to dominate (Salabei et al. 2013).

3 PI3Ks and the Vascular-Immune Connection

The involvement of PI3K signaling in a wide variety of immune cell types and inflammatory processes participating in vascular-related pathologies prompted intense research efforts, aimed at understanding the potential contribution of immune cell PI3K signaling in highly diffused pathologies such as hypertension and atherosclerosis. Leukocytes express all the members of the PI3K family but the majority of work on PI3K signaling in immune cells has been conducted on PI3Kδ and PI3Kγ, which are highly expressed within the hematopoietic compartment (Lupieri et al. 2015).

Among class I PI3K, the role of the γ-isoform, important in both smooth muscle and immune cells, revealed interesting functions at the crossroad of vascular-immune interfaces. By taking the advantage of PI3Kγ-deficient mice and mice expressing a catalytically inactive PI3Kγ, it was shown that the inhibition of PI3Kγ catalytic activity significantly reduced the emergency of atherosclerotic lesions in different mouse models susceptible to atherosclerosis (Chang et al. 2007; Fougerat et al. 2008). Further experiments clarified that the role exerted by PI3Kγ was mainly mediated by immune cells because chimeric mice with a selective ablation of PI3Kγ in hematopoietic-derived cells displayed a similar protection from atherosclerosis

progression (Fougerat et al. 2008). The investigation into cell-specific mechanisms underlying the role of PI3Kγ in atherosclerosis revealed a multitude of functions exerted by PI3Kγ in immune cells important for the development of arterial lesions. In the stage of foam cell formation, PI3Kγ plays a role in the internalization of oxidized low-density lipoprotein by macrophages in atherosclerotic plaques of ApoE-null mice and *p110γ*$^{-/-}$ mice lacking the catalytic subunit of PI3Kγ (Chang et al. 2007). At later stages, when atherosclerosis is also characterized by arterial lesions, PI3Kγ exerts additional roles in T cells, particularly relevant for the establishment of intimal hyperplasia after arterial injury. In fact, mice devoid of PI3Kγ activity and subjected to a hypercholesterolemic diet or mechanical arterial injury, display a marked defect in T cell infiltration (Fougerat et al. 2008; Smirnova et al. 2014) (Table 1). Additional studies revealed more specific functions of PI3Kγ in T cells. In particular, by reconstituting *Rag2* knock out mice—which are devoid of mature lymphocytes—with a pool of PI3Kγ-deficient CD4^{+} T cells, it was observed a protection against intimal hyperplasia, as compared to *Rag2* knock out reconstituted with wild-type CD4^{+} T cells (Smirnova et al. 2014). Conversely, no effect on T cell infiltration was observed (Smirnova et al. 2014). Taken together these data suggest that future studies will be necessary to completely unravel the molecular mechanisms leading to the recruitment of PI3Kγ and the signaling pathways downstream in T cells, in the context of vascular pathophysiology.

Another set of studies proposed a role for PI3Kγ in mediating Ca^{2+} signaling in T cells (Swat et al. 2006), whereby T cell receptor (TCR) activation would depend on pan-PI3K-mediated activation of the GDP/GTP exchange factors (GEF) Vav1, which in turn activates PLCγ by phosphorylation, to enhance Ca^{2+} influx (Reynolds et al. 2002). With a mechanism similar to that observed in SMCs, it has also been shown that exogenous PI(3,4,5)P3 activates Ca^{2+} influx in T cells, suggesting the possibility of a direct activation of Ca^{2+} channels mediated by PI(3,4,5)P3 and independent of TCR stimulation (Hsu et al. 2000). The increasing number of studies demonstrating a necessary and non-redundant role of T cells in vascular pathologies such as hypertension (Drummond et al. 2019; Carnevale 2020; Carnevale and Lembo 2020), coupled with previous observations highlighting a key role of PI3Kγ in high blood pressure and related target organ damage (Carnevale and Lembo 2012; Carnevale et al. 2012; Ghigo et al. 2017), suggest the necessity to further investigate potential PI3Kγ-related mechanisms at the vascular-immune connection (Table 1).

Even though most works have investigated the role of class I PI3K in T lymphocytes, some studies also proposed a role for class II PI3K-C2β in T cell activation (Cai et al. 2011; Srivastava et al. 2006, 2009). In this regard, it has been shown that TCR stimulation recruits PI3K-C2β, leading to KCa3.1 channel activation and subsequent Ca^{2+}-mediated T cell transcriptional program activation and cytokine production (Srivastava et al. 2009). While scarce data are available defining the role of class II and III PI3Ks in immune cells, the above results prompt further efforts in investigating this aspect.

The vascular milieu is also influenced by other immune cells, both at the steady-state and in disease. Among these cells, an involvement of mast cells in arterial chronic inflammation (Sun et al. 2007) and in experimental models of

cardiometabolic diseases (Bot et al. 2015; Shi et al. 2015) has been demonstrated and, interestingly, there is evidence that a crucial role in the above functions is played by PI3Kγ (Wymann et al. 2003). In particular, it has been shown that in bone marrow-derived mast cells isolated from PI3Kγ$^{-/-}$ mice and primed with allergens, the costimulatory potential of adenosine was abolished, inducing a massive influx of external Ca^{2+} (Laffargue et al. 2002). Nonetheless, the absence of PI3Kγ had no effect on the potential of adenosine to release Ca^{2+} from internal stores, further suggesting that PI3Kγ could directly modulate the opening of Ca^{2+} channels at the plasma membrane by generating PI(3,4,5)P3. Interestingly, the in vitro addition of exogenous PI(3,4,5)P3 to RBL-2H3 cells (basophilic leukemia cell line isolated from rat) revealed effects overlapping to those observed in smooth muscle and T cells, i.e., the capability to induce a massive influx of Ca^{2+} (Ching et al. 2001).

Another immune cell type participating in the modulation of vascular environment is B cells, crucial regulators of the humoral adaptive immune response. Mainly known to produce antibodies and cytokines and to participate in lymphoid tissue organization, B cells have also been involved in arterial pathology and, more specifically, in atherosclerosis. Mice devoid of the δ-isoform of class IA PI3Ks have less marginal zone B cells and B1 cells, manifesting a general defect in B cell development (Clayton et al. 2002; Jou et al. 2002; Okkenhaug et al. 2002) (Table 1). Since B cell subtypes have dual functions in the atherosclerotic process, with B2 B cells being atheroprone and B1-B cells being atheroprotective, a generalized inhibition of PI3Kδ might be undesirable, suggesting the need to investigate more selective isoform functions in this pathophysiological context.

4 Conclusions

PI3Ks and related intracellular signaling pathways are clearly essential for vascular homeostasis, being involved in the regulation of endothelial and SMCs functions. Several studies have highlighted a role of various members of the PI3Ks family in vascular diseases, making it conceivable that this class of enzymes represents a promising target for the treatment of cardiovascular diseases. Over the past years, a variety of mouse models have been developed to investigate the effect of selective inhibition of one or more isoforms in vascular pathology and related complications. Interestingly, a growing body of work clarified that the complexity of functions that PI3Ks family might fulfill depends also on the delicate relationship established between components of the vascular and immune systems. Although it has been generally considered that the immune system participates in the sequelae of events leading to vascular pathology, it is becoming increasingly clear that immune cells are also components of the vascular milieu at the steady-state. This emerging complexity endorses the necessity of further studies investigating the role of PI3Ks not only in relation to their isoform-specific functions but also to their cell-specific roles. New transgenic mouse models allowing cell-specific deletion of various PI3Ks and new

pharmacological inhibitors with enhanced selectivity will help to tackle this issue that, despite challenging, has enormous therapeutic perspectives.

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PI3K and AKT at the Interface of Signaling and Metabolism

Giovanni Solinas and Barbara Becattini

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Abstract The PI3K/AKT signaling module is recruited by several receptors implicated in maintaining tissue and metabolic homeostasis and signaling pathways controlling immune responses. Constitutive activation of PI3K/AKT signaling leads to tissue overgrowth and is frequently observed in cancer cells, whereas reduced PI3K/AKT signaling is associated with diabetes and growth defects. Thus, a critical roadblock to effective PI3K-targeted therapy comes from the crucial role of PI3K/AKT signaling in systemic metabolic homeostasis. This chapter describes the role of PI3K/AKT in insulin signaling and metabolic homeostasis and the interplay

G. Solinas (✉) · B. Becattini
The Wallenberg Laboratory for Cardiovascular and Metabolic Research, Department of Molecular and Clinical Medicine, University of Gothenburg, 41345 Göteborg, Sweden
e-mail: giovanni.solinas@wlab.gu.se

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between insulin action and metabolic feedback loops that cause resistance to PI3K-targeted therapies. Furthermore, we provide examples of insulin-independent roles for PI3K/AKT in metabolic homeostasis, and some generalizations on the action of PI3K/AKT signaling at the interface of signaling and metabolism are derived. Finally, the specific roles for different class I PI3K isoforms in controlling systemic metabolic homeostasis and energy balance are discussed. We conclude that defining the functional specificities and redundancies of different class I PI3K isoforms in pathways driving disease and controlling metabolic homeostasis is fundamental to develop novel PI3K-targeted therapies.

1 Introduction

The PI3K/AKT pathway is a pleiotropic signaling module implicated in a variety of signals controlling cell survival, proliferation, differentiation, many immune functions, and cell and systemic metabolic homeostasis (Burke and Williams 2015; Ghigo et al. 2012; Goncalves et al. 2018; Hawkins and Stephens 2015; Pompura and Dominguez-Villar 2018; Wymann and Solinas 2013). Hence, it is not possible to fully describe the role of the PI3K/AKT pathway at the interface of signaling and metabolism in one book chapter. Here we describe the physiological role and molecular mechanisms by which PI3K/AKT signaling controls systemic metabolic homeostasis, we discuss the relevance of these mechanisms to PI3K-targeted therapies, and present some conceptual generalizations.

2 PI3K/AKT Signaling Mediates Insulin Action in Metabolic Homeostasis

2.1 Insulin Integrates Glucose and Lipid Homeostasis

The PI3K/AKT signaling module is a potent regulator of systemic metabolic homeostasis, an action that is primarily related to its role in insulin signaling (Boucher et al. 2014; Bridges and Saltiel 2015; Czech 2017; Haeusler et al. 2018; Petersen and Shulman 2018; Saltiel 2016; Titchenell et al. 2017).

Pancreatic β-cells secrete insulin in response to high levels of blood glucose by a mechanism involving increased intracellular ATP levels, leading to the closure of K^+ channels and membrane depolarization. The change in membrane potential causes the opening of Ca^{2+} channels and increases the intracellular concentration of Ca^{2+}, which drives the secretion of insulin granules (Lefebvre et al. 1987; Rorsman and Ashcroft 2018; Rorsman and Braun 2013; Wollheim and Sharp 1981). However, glucose is not

the only molecule controlling insulin secretion, as glucose-stimulated insulin secretion is modulated by several extracellular signals, including the hormones somatostatin, glucagon, and glucagon-like peptide 1 (GLP-1); and neurotransmitters such as adrenaline and acetylcholine (Rorsman and Braun 2013). Furthermore, insulin secretion is also regulated by some amino acids, such as leucine (Newsholme et al. 2005), and by free fatty acids (Dobbins et al. 1998; Hauke et al. 2018; Itoh et al. 2003).

Overall, insulin is a signal maintaining glucose and lipid homeostasis, which is chiefly controlled by blood glucose levels, through a mechanism integrating information on amino acids and free fatty acid levels with hormonal and neuronal inputs. Elevated circulating levels of insulin potently suppress the endogenous production of glucose, which mostly occurs in the hepatocyte, and dampen circulating levels of glycerol and free fatty acids by suppressing adipose tissue lipolysis (Boucher et al. 2014; Czech 2017; Haeusler et al. 2018; Saltiel 2016; Titchenell et al. 2017). Furthermore, insulin increases glucose uptake in adipocytes and myocytes (Boucher et al. 2014; Czech 2017; Haeusler et al. 2018; Saltiel 2016; Titchenell et al. 2017).

Overall, insulin is a signal induced in response to elevated levels of glucose, some amino acids, and free fatty acids, and controls glucose and lipid metabolism by different molecular mechanisms recruited by the PI3K/AKT cascade.

2.2 PI3K/AKT Signaling is Essential for Insulin Action in Metabolism

The insulin receptor (IR) is a member of the receptor tyrosine kinase superfamily (RTKs). Insulin binding to the IR (Fig. 1a) activates IR intracellular tyrosine kinase activity, receptor autophosphorylation, and phosphorylation of the insulin receptor substrate proteins (IRS) 1 and 2 on multiple tyrosine residues (Fig. 1b) (Boucher et al. 2014). Tyrosine residues phosphorylated by the activated IR serve as binding sites for proteins containing SH2 domains, such as the adapter protein Grb2 (growth factor receptor-bound protein 2) that recruits the guanine nucleotide exchange factors SOS (son of sevenless) proteins, which activate the RAS/MAPK (mitogen-activated protein kinases) signaling pathway (Fig. 1c). However, to date, the RAS/MAPK signaling cascade has not been implicated in any of the metabolic effects of insulin, which instead are dependent on the PI3K/AKT signaling pathway (Boucher et al. 2014; Czech 2017; Haeusler et al. 2018; Petersen and Shulman 2018; Saltiel 2016). Insulin activates the PI3K signaling pathway through the binding of class IA PI3K-regulatory subunits p85α/β, p55α/γ, or p50α, via their SH2 (Src homology 2) domains to the phosphorylated tyrosine residues of IRS1-2 or of the IR (Ruderman et al. 1990) (Fig. 1d). Class IA PI3K regulatory subunits form stable dimers with PI3K catalytic subunits and inhibit PI3K activity. However, binding of PI3K regulatory subunits to tyrosine-phosphorylated peptides increases PI3K activity (Burke and Williams 2015) (Fig. 1d). An additional activation step for class I PI3K activity is given by direct

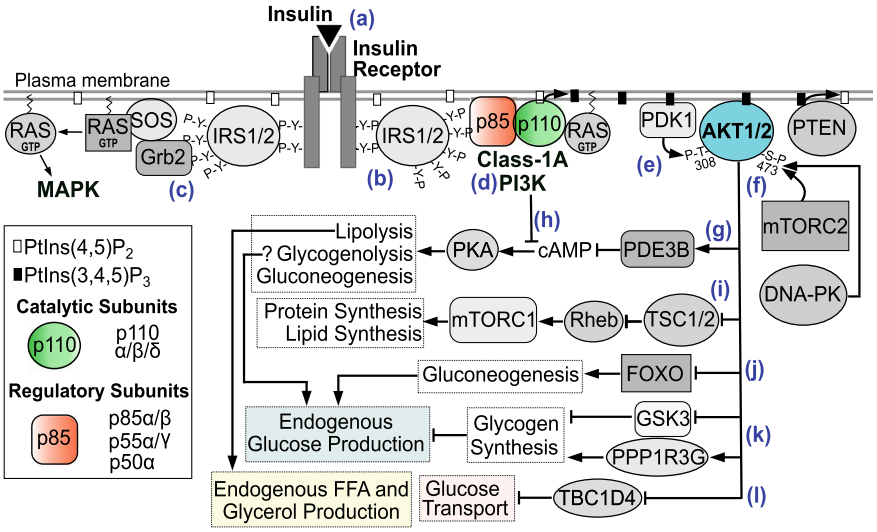


Fig. 1 The insulin signal transduction pathway. **a** Insulin binding to its receptor at the extracellular surface of the plasma membrane leads to the induction of its tyrosine kinase activity on the intracellular side. **b** Activated insulin receptor undergoes autophosphorylation and phosphorylates IRS1/2 proteins on multiple tyrosine residues, which serve as binding sites for SH2-domain-containing adapter proteins such as **c** Grb2 which induces the RAS/MAPK pathway, and **d** the regulatory subunits of class IA PI3Ks. The latter leads to the activation of the catalytic subunit of PI3K at the plasma membrane which may be further promoted by RAS binding to some catalytic PI3K subunits. The activated PI3K produces the membrane lipid PtIns(3,4,5)P_3 which acts as binding site for PH-domain containing proteins such as PDK1 and AKT. **e** This leads to the activation of AKT by direct phosphorylation by PDK1 at threonine 308. **f** Full activation of AKT is achieved by additional phosphorylation at serine 473 by mTORC2 and other kinases such as DNA-PK. PI3K/AKT signaling has an antagonistic action to cAMP/PKA signaling by **g** a mechanism involving direct phosphorylation of PDE3B by AKT and via **h** another mechanism that is PI3K-dependent but AKT-independent. **i** AKT induces mTORC1 activity, which is important for protein and lipid synthesis, by direct phosphorylation of TSC2. **j** AKT inhibits FOXO transcription factors controlling genes involved in gluconeogenesis, and **k** AKT promotes glycogen synthesis by phosphorylation of GSK3 and PPP1R3G. Finally, **l** AKT phosphorylates TBC1D4, leading to increased glucose transport into muscle and fat

binding of some PI3K catalytic subunits to RAS (Buckles et al. 2017; Siempelkamp et al. 2017; Vadas et al. 2011) (Fig. 1d). RAS promotes insulin-induced AKT phosphorylation in the hepatocyte but is largely dispensable for insulin signaling downstream AKT (Molinaro et al. 2019). Once activated, class IA PI3K phosphorylate the plasma membrane lipid PtdIns(4,5)P_2 (phosphatidylinositol-4,5-bisphosphate) at position 3 to generate PtdIns(3,4,5)P_3 (phosphatidylinositol-3,4,5-bisphosphate) (Fig. 1d). The kinases PDK1 (3-phosphoinositide-dependent protein kinase 1) and AKT bind to PtdIns(3,4,5)P_3 via their PH (pleckstrin homology) domains, leading to the phosphorylation of AKT at threonine 308 by PDK1 (Alessi et al. 1997), an essential step in AKT activation (Hemmings and Restuccia 2012) (Fig. 1e). Full activation of AKT is achieved by additional phosphorylation of AKT at serine 473 by

mTORC2 (mechanistic target of rapamycin complex 2) (Sarbassov et al. 2005), and other kinases such as DNA-PK (DNA protein kinase) (Feng et al. 2004) (Fig. 1f). The activation of AKT is fundamental for insulin actions in metabolic homeostasis as most of the known metabolic effectors of insulin signaling are regulated by AKT (Boucher et al. 2014; Czech 2017; Haeusler et al. 2018; Petersen and Shulman 2018; Saltiel 2016). A major mechanism by which PI3K/AKT controls lipid homeostasis depends on its antagonism with cAMP/PKA signaling, which is the pathway driving adipose tissue lipolysis (Czech et al. 2013; Zechner et al. 2017). It was shown that AKT directly phosphorylates and activates the phosphodiesterase PDE3B, leading to increased degradation of cAMP (Eriksson et al. 1995; Kitamura et al. 1999; Rahn et al. 1994) (Fig. 1g). However, it was proposed that insulin-mediated suppression of lipolysis may involve a mechanism that is PI3K-dependent but AKT-independent (Choi et al. 2010; DiPilato et al. 2015) (Fig. 1h).

Overall it can be concluded that in the adipocyte there is a potent antagonistic action between insulin/PI3K/AKT signaling and cAMP/PKA-driven lipolysis by a mechanism that is only partially understood. It is worthy of note that cAMP/PKA signaling also mediates the effects of glucagon on glycogenolysis and glucose production in the hepatocyte (Ravnskjaer et al. 2016; Rui 2014). Therefore, the antagonistic crosstalk between the PI3K/AKT and cAMP/PKA signaling controls the release of free fatty acids from the adipocyte and glucose release from the hepatocyte. Another metabolically relevant target of AKT is TSC2 (Tuberous Sclerosis Complex 2), which is inactivated by AKT via direct phosphorylation (Inoki et al. 2002) (Fig. 1i). TSC2 is a negative regulator of mTORC1 (mechanistic target of rapamycin complex 1), which is an important mediator of the effects of insulin on protein and lipid synthesis (Liu and Sabatini 2020; Shimobayashi and Hall 2014). AKT also phosphorylates the transcription factor Forkhead box protein O (FOXO), inducing FOXO translocation from the cell nucleus to the cytoplasm, thereby reducing the expression of genes driving endogenous glucose production, such as glucose-6 phosphatase (Brunet et al. 1999; Dong et al. 2008; Matsumoto et al. 2007; Nakae et al. 1999) (Fig. 1j). Another mechanism by which insulin suppresses endogenous glucose production is by promoting glucose storage to glycogen, through direct phosphorylation of the enzyme GSK3 (glycogen synthase kinase 3) by AKT (Cross et al. 1995) (Fig. 1k). GSK3 is a negative regulator of the enzyme glycogen synthase, and GSK3 phosphorylation by AKT reduces GSK3 activity, which increases glycogen synthesis and reduces glucose production (Cross et al. 1995). Insulin also promotes glycogen synthase activity via AKT-mediated phosphorylation of PPP1R3G (Protein phosphatase 1 regulatory subunit 3G), by promoting glycogen synthase dephosphorylation (Fig. 1k) (Li et al. 2019b). Finally, the effects of insulin on increased glucose uptake in skeletal muscle and adipose tissue are also dependent on PI3K/AKT signaling, by a mechanism involving direct inhibitory phosphorylation of the Rab GTPase TBC1D4 by AKT, leading to increased translocation of the glucose transporter GLUT4 to the plasma membrane (Sano et al. 2003) (Fig. 1l).

Altogether, it can be concluded that all the known molecular mechanisms mediating the action of insulin in glucose and lipid metabolism are activated via the induction of PI3K signaling.

2.3 PI3K/AKT Signaling Controls Pancreatic β-Cell Mass and Function

Mice lacking the IR in their β-cells develop defective insulin secretion and glucose intolerance (Kulkarni et al. 1999), whereas mice lacking the IR and the IGF-1 (insulin-like growth factor-1) receptor became diabetic at three weeks of age due to a pronounced loss of β-cell mass (Ueki et al. 2006). The effects of insulin and IGF-1 on the mass and function of pancreatic β-cells are primarily mediated by PI3K/AKT signaling. Mice expressing a constitutively active AKT1 kinase in their pancreatic β-cells display improved glucose tolerance and insulin secretion and a larger pancreatic β-cells mass (Bernal-Mizrachi et al. 2001; Tuttle et al. 2001). Furthermore, mice lacking PDK1 in their pancreatic β-cells showed a reduced β-cell mass and developed hyperglycemia (Hashimoto et al. 2006). Moreover, mice with a genetic deletion of *Pik3r1* in their β-cells and with a systemic deletion of *Pik3r2* developed glucose intolerance because of defective glucose-stimulated insulin secretion and reduced β-cell mass (Kaneko et al. 2010).

Collectively these data indicate that insulin/IGF1-driven PI3K/AKT signaling plays a fundamental role in the maintenance of pancreatic β-cell mass and function. However, it was also shown that acute exposure of human islets to an insulin-mimetic reduced glucose-stimulated insulin secretion in a PI3K-dependent manner (Persaud et al. 2002), indicating that insulin signaling plays a negative feedback regulation on insulin secretion via PI3K. This idea is consistent with other studies showing that acute PI3K inhibition in isolated pancreatic β-cells potentiates glucose-stimulated insulin secretion (Aoyagi et al. 2012; Eto et al. 2002; Kolic et al. 2013; Zawalich and Zawalich 2000).

Cell culture studies indicate that PI3Kγ promotes insulin secretion in response to glucose and glucose-dependent insulinotropic polypeptide (GIP) by maintaining functional insulin secretory granules and promoting insulin exocytosis (Li et al. 2006; Pigeau et al. 2009). However, it is established that loss of PI3Kγ improves glucose tolerance in obese mice by increasing insulin sensitivity (Becattini et al. 2011; Breasson et al. 2017; Kobayashi et al. 2011; Torres et al. 2019). Furthermore, loss of PI3Kγ improved β-cell mass and function in the db/db model of obesity-driven diabetes (Breasson et al. 2018). Thereby, loss of PI3Kγ does not appear to significantly impair β-cell function in vivo.

Altogether these studies indicate that insulin/IGF1-driven PI3K/AKT signaling is necessary to maintain and expand β-cell mass and function. However, insulin-driven PI3K signaling also mediates acute negative feedback on insulin secretion.

3 The Role of Insulin/PI3K/AKT Signaling in PI3K Targeted Therapies

3.1 Iatrogenic Diabetes Dampens the Efficacy of Pan-PI3K Inhibition

The dependency on PI3K/AKT signaling for insulin action in metabolic homeostasis has a significant impact on the efficacy of PI3K-targeted therapies. PI3K/AKT signaling is frequently activated in cancer and plays a key role in tumor promotion (Goncalves et al. 2018; Janku et al. 2018; Vogelstein et al. 2013). Several small-molecule inhibitors targeting this pathway were tested as cancer therapies in the clinic (Goncalves et al. 2018; Janku et al. 2018; Vogelstein et al. 2013). Idelalisib, Copanlisib, Duvelisib, and Umbralisib have been approved for combination therapies of specific relapsed hematological malignancies (Das 2018; Dhillon and Keam 2021; Janku et al. 2018), and Alpelisib, a PI3K inhibitor with selectivity for PI3Kα, has been approved for combination therapy of HR-positive HER-negative advanced metastatic breast cancer-bearing PI3Kα activating mutations (Markham 2019). These results, although positive, did not match the high expectations on PI3K-targeted cancer therapy (Fruman et al. 2017; Janku et al. 2018; Okkenhaug et al. 2016; Thorpe et al. 2015). The difficulty of developing effective pan-PI3K inhibitors for the treatment of solid tumors as single therapeutics depends on the central role of PI3K/AKT signaling in the metabolic action of insulin (Fruman et al. 2017; Goncalves et al. 2018; Hopkins et al. 2020, 2018). High blood glucose levels lead to insulin secretion, which dampens hepatic glucose production, restoring normal glycemia and thereby reducing insulin levels (Fig. 2a). In obese insulin-resistant subjects, hyperinsulinemia may sustain PI3K/AKT signaling in insulin-sensitive tumors and promote tumor growth and progression (Fig. 2a). Indeed, hyperinsulinemia is an independent risk factor for some cancers (Anveden et al. 2017; Perseghin et al. 2012). Administration of pan-PI3K inhibitors causes severe hyperglycemia due to reduced PI3K signaling in metabolically relevant insulin target cells, such as the hepatocyte (Hopkins et al. 2018; Janku et al. 2018; Khan et al. 2016) (Fig. 2b). The elevated blood glucose levels stimulate insulin secretion by pancreatic β-cells leading to hyperinsulinemia, which partially reactivates PI3K/AKT signaling in the hepatocyte, but also in insulin-sensitive tumors (Hopkins et al. 2018). Thus, the hyperglycemia-hyperinsulinemia-PI3K loop drives resistance to pan-PI3K inhibitors, limiting their therapeutic application to the treatment of insulin-insensitive tumors (Fig. 2b) (Hopkins et al. 2020). Because AKT inhibitors also cause hyperglycemia (Crouthamel et al. 2009), this mechanism is expected to be also relevant for AKT-targeted therapies.

Targeting specific PI3K isoforms whose activity is dispensable for insulin action in metabolic homeostasis may avoid the deleterious side effects of pan-PI3K inhibitors on insulin signaling. Thus, a better understanding of specific and redundant functions of different class I PI3K at the interface between cell signaling and metabolism may lead to more effective PI3K-targeted therapies.

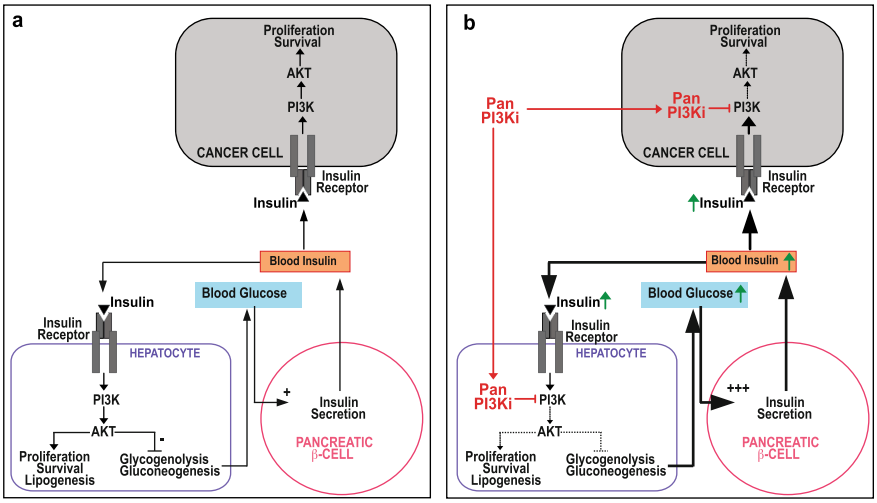
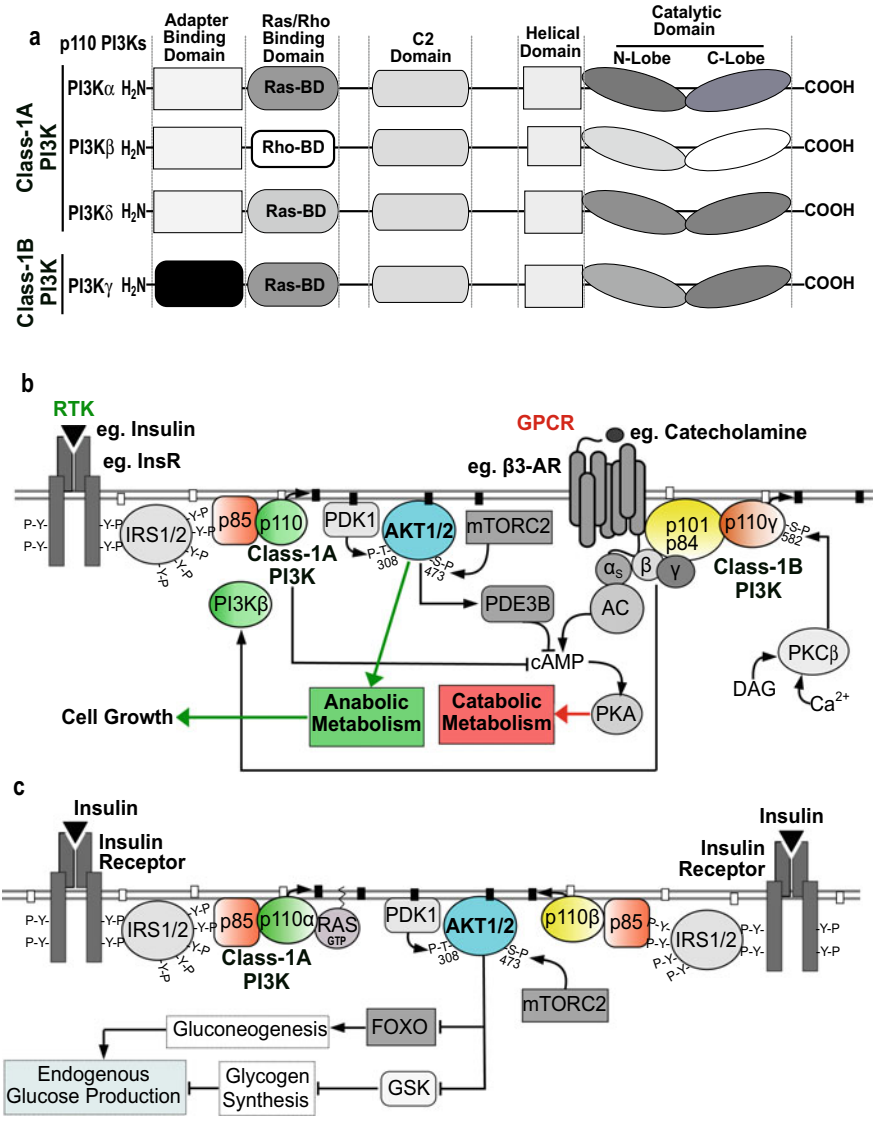


Fig. 2 The role of insulin/PI3K/AKT signaling in cancer risk and PI3K-targeted cancer therapy. **a** High blood glucose levels induce insulin secretion by the pancreatic β-cell; insulin suppresses endogenous glucose production by a mechanism dependent on the activation of the IR/PI3K/AKT signaling cascade in the hepatocyte. This creates a feedback loop linking PI3K activity in the hepatocyte and insulin-sensitive cells (e.g., adipocytes and myocytes) with glycemia and insulinemia. Circulating insulin levels are expected to drive PI3K/AKT signaling in insulin-sensitive cancer cells which do not have an oncogenic activation of the PI3K/AKT cascade and some studies indicate that chronic hyperinsulinemia increases the risk for some cancers. **b** In PI3K-targeted cancer therapy systemic administration of pan-PI3K inhibitors reduces PI3K activity in cancer cells but also in cells mediating insulin action on blood glucose (e.g., the hepatocyte); this causes severe hyperglycemia as adverse effect which in turn leads to a marked hyperinsulinemia. These supraphysiological levels of insulin caused by pan-PI3K inhibitors reactivate PI3K/AKT signaling in the hepatocyte and in insulin-sensitive cancer cells, thereby limiting the extent to which PI3K/AKT signaling can be effectively inhibited in these cells

3.2 Specificities and Redundancies of Class I PI3Ks in Cell Signaling

One prominent feature determining specificity and redundancy of catalytic PI3K subunits within a particular cell type is their different expression pattern. PI3Kα and PI3Kβ are ubiquitously expressed, whereas PI3Kγ and PI3Kδ are most abundant in hematopoietic cells (Burke and Williams 2015; Thorpe et al. 2015; Vanhaesebroeck et al. 2010).

Class I PI3K catalytic subunits PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ share a similar architecture comprising an N-terminal adapter binding domain, a RAS-binding domain; a C2 domain; a helical domain, and a bilobular catalytic domain (Fig. 3a). However, PI3K catalytic subunits also display distinct structural features conferring some specificity. PI3Kα, PI3Kβ, and PI3Kδ have an adapter binding domain that mediates a stable interaction with the class IA PI3K regulatory subunits p85α/β,



p55α/γ, and p50α (Burke and Williams 2015; Rathinaswamy and Burke 2020). Class IA regulatory subunits inhibit the activity of the associated catalytic subunits and have two SH2 domains that can bind to phosphorylated tyrosine motifs pYXXM, such as the one on the insulin receptor substrates (Figs. 1 and 3). The binding of the class IA PI3K regulatory subunits to phosphorylated tyrosines disrupts their inhibitory

◀**Fig. 3** Specificities and redundancies define the role for each class I PI3K in metabolism. **a** The four class I PI3K catalytic subunits display a similar architecture with specific features. The adapter binding domain distinguishes the class IA PI3Kα, PI3Kβ, and PI3Kδ from the class IB PI3Kγ. Class IA PI3K catalytic subunits form dimers with class IA regulatory subunits (p85α/β, p55α/γ, and p50α), which link class IA PI3Ks to RTK signaling, whereas the class IB catalytic PI3Kγ dimerizes with specific class IB regulatory subunits (p101, p84/p87) that mediate the activation of PI3Kγ downstream GPCRs. Another major difference is in the RAS-binding domain of PI3Kβ, which differs from all the other class I PI3Ks in that it does not bind to RAS GTPases but instead is recruited by RHO-GTPases. The different class I PI3K catalytic subunits display unique characteristics in their catalytic domains, which may confer different levels of activity. Specific features of the catalytic domains have been exploited to develop PI3K inhibitors with isoform-selectivity. **b** p85-like regulatory subunits mediate the activation of class IA PI3K downstream receptor tyrosine kinases (RTK) such as the insulin receptor and the insulin-like growth factor receptor-1; p101 and p84/p87 link PI3Kγ to G-protein coupled receptors (GPCR) such as β-adrenergic receptors. PI3Kβ has the peculiar ability to be also activated by GPCR by a mechanism that remains unresolved and may play a unique role in transducing coincident signals from GPCR and RTK. The crosstalk between different RTK and GPCR is highly relevant to metabolic homeostasis as insulin control of lipolysis in the adipocyte operates in opposition to catecholamine, and insulin action on hepatic glucose production operates in opposition to glucagon. The adrenergic receptors and the glucagon receptors are GPCR. However, the role of different class I PI3K in the crosstalk between insulin, catecholamine, and glucagon remains largely unresolved. **c** Insulin signaling in the hepatocyte controlling endogenous glucose production is mediated by redundant PI3Kα and PI3Kβ activities, whereas PI3Kδ and PI3Kγ activities are dispensable for insulin signaling in the hepatocyte. These redundancies and specificities may be exploited to develop PI3K-targeted therapies with optimal therapeutic index using isoform-selective inhibitors which do not interfere with insulin action in metabolic homeostasis

action on the associated PI3K catalytic subunits, leading to PI3K activation (Burke and Williams 2015).

The class IB PI3Kγ catalytic subunit has a different adapter binding domain that interacts with specific regulatory subunits (p101 and p87/p84). The domain architecture of p101 and p87/p84 remains unresolved, and these regulatory subunits do not directly bind to tyrosine-phosphorylated proteins but interact with the βγ subunit of trimeric G-proteins following the activation of G-protein coupled receptors (Fig. 3a, b) (Burke and Williams 2015; Rathinaswamy and Burke 2020; Vanhaesebroeck et al. 2010). Each catalytic class I PI3K (PI3Kα, PI3Kβ, and PI3Kδ) can dimerize with each class IA regulatory subunit (p85α, p85β, p55α, p55γ, and p50α) to form up to fifteen distinct enzymatic complexes, which may contribute to defining functional specificities and redundancies. Indeed, a study reported that PI3Kδ is preferentially associated with p85α compared to p85β, whereas PI3Kα and PI3Kβ do not appear to discriminate between p85α and p85β regulatory subunits (Tsolakos et al. 2018). Functional specificity of regulatory subunits was demonstrated for the class IB PI3Ks, with p101-PI3Kγ complex being most important to GPCRs signaling, whereas the p84-PI3Kγ complex is key to IgE/antigen receptor-induced mast cell degranulation (Bohnacker et al. 2009; Rathinaswamy and Burke 2020; Shymanets et al. 2013; Vadas et al. 2013).

An additional level of specificity of PI3K catalytic subunits is given by their RBD (RAS-binding domain), as PI3Kα, PI3Kδ, and PI3Kγ activities are enhanced by

RAS GTPases, whereas PI3Kβ is insensitive to RAS-GTPase, but is activated by RHO-GTPases (Fig. 3a) (Fritsch et al. 2013).

Another feature of PI3Kβ is that, differently from PI3Kα and PI3Kδ but similarly to PI3Kγ, it is activated by the βγ subunits of trimeric G-proteins by a mechanism that remains unresolved (Kurosu et al. 1997) (Fig. 3b). This feature distinguishes PI3Kβ from other class IA PI3Ks, as PI3Kβ can transduce signals redundantly with PI3Kγ downstream specific GPCR (Guillermet-Guibert et al. 2008), and redundantly with PI3Kα downstream the insulin receptor (Molinaro et al. 2019). However, PI3Kβ may also play a unique role downstream concomitant GPCRs and RTKs signals (Houslay et al. 2016).

Finally, PI3Kγ can be activated by direct phosphorylation at serine 582 by PKCβ, which correlates with the dissociation of PI3Kγ from the p84 regulatory subunit (Walser et al. 2013) (Fig. 3b).

It should also be considered that different catalytic PI3K isoforms may have different catalytic potency and thereby may generate signals of different amplitude (Madsen and Vanhaesebroeck 2020). Measurements of PI3Kα and PI3Kβ activities by in vitro kinase assays indicate that PI3Kα has a superior catalytic activity (Beeton et al. 2000; Meier et al. 2004; Utermark et al. 2012). It was also proposed that the less active PI3Kβ may reduce PI3K/AKT signaling by competing with the more active PI3Kα along the same signaling cascade (Thorpe et al. 2015; Utermark et al. 2012). However, it is unclear to what extent differences in PI3K activities measured in vitro depend on the in vitro kinase assay being better optimized for a specific isoform (e.g., PI3Kα). Quantitative measurements of activities of different class I PI3K in intact cells are necessary to address this issue.

The features described above provide insights on redundant and specific roles of class I catalytic PI3K subunits downstream RTK and GPCR signaling (Fig. 3b) and in metabolic homeostasis. Indeed, insulin actions in adipose tissue lipolysis and hepatic glucose production operate in opposition to catecholamine and glucagon signaling, which are mediated by GPCRs. Redundancies of PI3K activities in insulin action such as the one between PI3Kα and PI3Kβ in hepatic insulin signaling (Fig. 3c) (Molinaro et al. 2019) may indicate isoform-specific PI3K-targeted therapies with optimal therapeutic index.

4 PI3K/AKT Signaling Drives Anabolic Metabolism and Cell Growth

Signaling dynamics (amplitude, length, and frequency) play a major role in determining the physiological outcome of a signaling cascade (Kholodenko et al. 2010; Madsen and Vanhaesebroeck 2020; Solinas and Becattini 2017). A good generalization for PI3K/AKT signaling in metabolism is that the outcome of a robust and prolonged PI3K/AKT activation is the induction of anabolic metabolism and cell growth (Fig. 3b).

Insulin action in anabolic metabolism is mediated by PI3K/AKT signaling (Boucher et al. 2014; Czech 2017; Fruman et al. 2017; Goncalves et al. 2018; Hopkins et al. 2020; Petersen and Shulman 2018; Saltiel 2016) and loss of insulin or AKT signaling in the adipocyte, a cell type most sensitive to insulin, leads to adipose tissue atrophy (Shearin et al. 2016; Softic et al. 2016). Insulin is not the only hormone driving anabolic metabolism through PI3K/AKT signaling, and an outstanding example is insulin-like growth factor-1 (IGF-1). IGF-1 receptor (IGF-1R) signaling via PI3K/AKT is closely linked to anabolic metabolism and cell growth, driving the increase of muscle mass induced by physical exercise (Cohen et al. 2015; Hawley et al. 2014; Vainshtein and Sandri 2020). Loss of class IA PI3K activity in myocytes causes defective muscle growth, glucose intolerance, and elevated blood lipids (Luo et al. 2006). Furthermore, ablation of AKT1 and AKT2 in mice causes severe muscle atrophy (Peng et al. 2003). Conversely, activation of PI3K/AKT signaling in myocytes causes muscle fiber hypertrophy (Bodine et al. 2001; Izumiya et al. 2008; Lai et al. 2004; Pallafacchina et al. 2002).

The powerful effects of sustained PI3K/AKT signaling on anabolic cell metabolism and cell growth in humans are illustrated by a family of tissue overgrowth syndromes caused by genetic mutations activating PI3K/AKT signaling (Keppler-Noreuil et al. 2016; Madsen et al. 2018; Martinez-Lopez et al. 2017). Indeed, genetic mutations of the *PIK3CA* gene leading to a constitutive active PI3Kα, genetic activation of AKT, or loss of function mutations of PTEN, TSC1, and TSC2 are the cause of a group of genetic diseases with distinct features but all leading to overgrowth of the mutated cell (Keppler-Noreuil et al. 2016; Madsen et al. 2018; Martinez-Lopez et al. 2017).

PI3K/AKT signaling can be induced in all types of cells by virtually any receptor with a tyrosine kinase activity and by G-protein coupled receptors (Burke and Williams 2015). Thus PI3K/AKT is a universal and versatile signaling module controlling anabolic cell metabolism (Fig. 3b).

The action of PI3K/AKT signaling in anabolic metabolism is mediated by the targets downstream AKT described in Fig. 1 and, at least in some contexts, operates in antagonism with cAMP/PKA signaling inducing mobilization of intracellular energy storage (Fig. 3b) (Ravnskjaer et al. 2016). Two outstanding examples of this crosstalk are the antagonistic actions of insulin/PI3K/AKT signaling and norepinephrine/cAMP/PKA signaling on lipolysis in the adipocyte (Czech et al. 2013; Zechner et al. 2017); and the antagonistic actions of insulin/PI3K/AKT signaling and glucagon/cAMP/PKA signaling on glycogenolysis in the hepatocyte (Jiang and Zhang 2003; Rui 2014). Overall, although the action of PI3K/AKT in systemic metabolic homeostasis is largely dependent on its role in insulin signaling, the role of PI3K/AKT in cell metabolism should be considered in a broader context.

5 Insulin and PI3K/AKT Signaling Promote Obesity

β-adrenergic signaling is a most potent signaling pathway inducing lipolysis in the white adipocyte (Zechner et al. 2017). Sustained β-adrenergic/cAMP/PKA signaling induces the formation and activation of thermogenic beige adipocytes, which burn lipids and glucose at an exceptionally high rate to generate heat (thermogenesis) (Chouchani et al. 2019; Cohen and Spiegelman 2016; Solinas et al. 2015). Notably, low β-adrenergic-driven lipolysis in adipocytes predicts long-term weight gain and high blood glucose in humans (Arner et al. 2018).

The most potent negative regulator of β-adrenergic-driven cAMP/PKA signaling and lipolysis is insulin via PI3K/AKT signaling (Fig. 3b) (Zechner et al. 2017). Hence, insulin is a powerful signal opposing lipid mobilization from adipose tissue (Zechner et al. 2017), which may play an important role in the development of obesity (Kolb et al. 2018). In support of this view, preclinical studies indicate that reducing PI3K/AKT signaling by decreasing insulinemia, by overexpression of PTEN, or PI3K pharmacological inhibition protected mice from excessive adiposity (Garcia-Cao et al. 2012; Lopez-Guadamillas et al. 2016; Mehran et al. 2012; Ortega-Molina et al. 2012; Ortega-Molina et al. 2015; Page and Johnson 2018; Templeman et al. 2017).

Overall the studies described above indicate that insulin/PI3K/AKT signaling plays a significant role in promoting diet-induced obesity. Pan-PI3K inhibition reduces adiposity and insulin resistance in mouse and monkey models of obesity but also causes severe acute hyperglycemia and hyperinsulinemia (see also Fig. 2b).

A better understanding of the role of different class I PI3Ks in insulin action, in glucose homeostasis, and lipid metabolism may lead to more effective antiobesogenic therapies targeting selected PI3K isoforms.

6 PI3K/AKT Signaling in Neurons Controls Metabolic Homeostasis

Insulin and leptin induce PI3K/AKT signaling in different types of neurons (Hausen et al. 2016; Klockener et al. 2011; Niswender et al. 2003; Xu et al. 2005). Central insulin signaling induces anorexia (Obici et al. 2002a), and inhibition of PI3K/AKT signaling within the hypothalamic arcuate nucleus blunts the effects of insulin on food intake (Niswender et al. 2003). It was also shown that hypothalamic insulin and leptin signaling contribute to the regulation of the suppression of hepatic glucose production (German et al. 2009; Obici et al. 2002b), and PI3K/AKT signaling in proopiomelanocortin (POMC) hypothalamic neurons was shown to contribute to the control of glucose homeostasis (Hill et al. 2009). Furthermore, PI3K/AKT signaling in different neuronal populations was shown to play powerful and, at times, opposite actions on energy balance. Constitutive activation of PI3K/AKT signaling in POMC neurons by ablating the phosphatase PTEN specifically in these cells made

mice more sensitive to diet-induced obesity (Plum et al. 2006). Conversely, constitutive activation of PI3K/AKT signaling in ObRb (leptin receptor)-expressing neurons by ablating PTEN in these cells led to a leaner phenotype by driving browning of white adipose tissue and increasing energy expenditure (Plum et al. 2007). Elevated PI3K/AKT signaling in SF-1 (steroidogenic factor 1) neurons of the ventromedial hypothalamus (VMH) induced by ablation of PTEN specifically in these cells, led to increased food intake and adiposity (Klockener et al. 2011). Furthermore, knockout of IR in these cells caused a leaner phenotype and reduced food intake (Klockener et al. 2011). However, in another study, loss of PI3Kα in SF-1 VMH neurons predisposed mice to obesity by reducing energy expenditure and blunting the anorexigenic effects of leptin (Xu et al. 2010).

The notion that PI3K/AKT signaling within different types of neurons plays opposite effects on energy balance is further supported by a study where PI3Kα and PI3Kβ were specifically ablated in POMC neurons and AgRP (agouty related peptide) neurons (Al-Qassab et al. 2009). In this study, loss of PI3Kα in either POMC or AgRP neurons did not significantly affect adiposity. However, PI3Kβ ablation in POMC neurons caused leptin resistance and promoted adiposity, whereas PI3Kβ ablation in AgRP neurons caused a lean phenotype (Al-Qassab et al. 2009).

Altogether, the studies above have implicated PI3K/AKT signaling in insulin and leptin action within the central nervous system controlling endogenous glucose production, energy balance, and adiposity. However, the effects of PI3K/AKT signaling on energy balance depend on the specific type of neuron where the PI3K/AKT pathway is activated.

7 Role of Specific Catalytic PI3K Subunits in Insulin Signaling and Obesity

7.1 PI3Kα is a Major but not the Only Mediator of Insulin action in Metabolism

Early studies investigating the role of specific PI3K catalytic subunits in insulin action led to the idea that PI3Kα is chiefly responsible for insulin-induced PI3K/AKT signaling controlling metabolic homeostasis, with other PI3K catalytic subunits playing a marginal role in this process (Foukas et al. 2006; Knight et al. 2006). These studies are based on PI3K inhibitors with some selectivity against specific isoforms (Knight et al. 2006) and mice with a heterozygous loss of function mutation of PI3Kα (PI3Kα$^{D933A/+}$) (Foukas et al. 2006). However, PI3Kα$^{D933A/+}$ mice improved their metabolic phenotype with time, indicating compensatory mechanisms (Foukas et al. 2013).

Further evidence supporting the idea of a role of PI3Kα in insulin signaling comes from studies in mice with tissue-specific deletion of PI3Kα. One study investigating mice with a conditional deletion of PI3Kα in the hepatocyte reported that these mice

developed some insulin resistance which worsened with aging (Sopasakis et al. 2010). An independent study showed that mice lacking PI3Kα in their hepatocytes display normal glucose tolerance when kept on chow-diet but developed glucose intolerance when placed on a high-fat diet (Chattopadhyay et al. 2011). Furthermore, these mice were protected from fatty liver induced by an obesogenic diet, indicating an essential role for PI3Kα in this process (Chattopadhyay et al. 2011).

Mice lacking PI3Kα in their adipocytes showed mildly enhanced β-adrenergic signaling in adipose tissue and accumulated less fat mass during aging, but adipose tissue development and circulating levels of free fatty acids were overall normal and AKT phosphorylation was not reduced in most fat pads (Araiz et al. 2019).

Overall, these studies indicate that PI3Kα is an important player in insulin signaling. However, the phenotypes described for mice lacking PI3Kα in adipocytes or hepatocytes are mild compared to the phenotype of mice lacking either the insulin receptor or AKT1 and AKT2 in these cells (Michael et al. 2000; Shearin et al. 2016; Softic et al. 2016; Titchenell et al. 2016; Wang et al. 2016), or mice expressing a PI3K dominant-negative adaptor in hepatocytes (Miyake et al. 2002).

Furthermore, mice with selective ablation of PI3Kα in myocytes showed reduced muscle mass with aging and about a 50% reduction of insulin-induced AKT phosphorylation in quadriceps (Li et al. 2019a).

Overall, a large body of evidence indicates that PI3Kα plays an important role in insulin signaling. Early studies led to the idea that the PI3K activity recruited by the active insulin receptor is virtually all from PI3Kα. However, selective ablation of PI3Kα does not lead to a severe metabolic outcome, which is expected for a complete blockade of insulin/PI3K/AKT signaling, and residual insulin-induced AKT phosphorylation is often observed in tissues lacking PI3Kα activity. Thus, from the studies above cannot be concluded that the PI3Kα is the only mediator of insulin-driven PI3K/AKT signaling, but appears to be redundant with the activity of another PI3K isoform.

7.2 PI3Kβ Activity Plays an Important Role in Growth and Metabolism

Acute inhibition PI3Kβ, in isolation, does not affect insulin signaling (Knight et al. 2006). Mice expressing a catalytically inactive form of PI3Kβ (PI3Kβ-KR) were viable but showed a markedly reduced body weight at weaning (Ciraolo et al. 2008). By the age of six months, PI3Kβ-KR mice developed insulin resistance and showed a less sustained insulin-induced AKT phosphorylation in their livers compared to control mice (Ciraolo et al. 2008). However, the metabolic phenotype of these mice could be at least in part consequent to the markedly reduced body weight at birth (Ciraolo et al. 2008). Furthermore, mice lacking PI3Kβ in adipose tissue (Araiz et al. 2019) or skeletal muscle (Li et al. 2019a) did not show an obvious metabolic phenotype.

Mice lacking PI3Kβ in hepatocytes showed reduced phosphorylation of AKT in response to insulin and developed mild glucose intolerance, although the authors could not find an effect of PI3Kβ blockade on IRS1-associated PI3K activity (Chattopadhyay et al. 2011). In another study, PI3Kβ was ablated from the liver using Cre-LoxP recombination, where the transgenic Cre recombinase is delivered using an adenovirus vector (Jia et al. 2008). Using this approach, the authors could not find an effect of PI3Kβ ablation on insulin-induced AKT phosphorylation, but these mice showed increased mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK) and developed some insulin resistance, but not hyperglycemia (Jia et al. 2008).

Further insights on a possible role for PI3Kβ in insulin signaling come from mice with heterozygous ablation of either PI3Kα, PI3Kβ, or both isoforms (Brachmann et al. 2005). Homozygous mutations leading to ablation of either PI3Kα or PI3Kβ cause early embryonic lethality (Bi et al. 2002, 1999), whereas heterozygous mice for PI3Kα or PI3Kβ loss of function appear normal and showed normal glycemic control and insulin sensitivity (Brachmann et al. 2005). However, heterozygous mice for loss of function mutations of both PI3Kα and PI3Kβ showed mild glucose intolerance and higher than control levels of insulin during a glucose tolerance test (Brachmann et al. 2005).

Collectively, the studies above indicate a possible role for PI3Kβ in glucose homeostasis. However, except for one study (Ciraolo et al. 2008), most works concluded that insulin-driven PI3K/AKT signaling is virtually entirely mediated by PI3Kα, with PI3Kβ playing a marginal role in this signaling pathway.

7.3 Insulin Signaling in the Hepatocyte and Hepatic Glucose Production Depend on Redundant PI3Kα and PI3Kβ Activities

The role of different PI3K catalytic subunits in the hepatocyte, the cell type responsible for most of the insulin action on endogenous glucose production, was recently solved (Molinaro et al. 2019). This study shows that hepatocyte-specific ablation of PI3Kα causes mild insulin resistance and hyperinsulinemia but not hyperglycemia and has no effects on insulin-driven AKT phosphorylation in primary mouse hepatocytes. Pharmacological mapping with isoform-selective PI3K inhibitors in primary hepatocytes from wild-type mice and mice lacking PI3Kα in hepatocytes shows that insulin-induced AKT phosphorylation in mouse hepatocytes depends on redundant PI3Kα and PI3Kβ activities (Molinaro et al. 2019). The role of PI3Kβ in insulin signaling within the hepatocyte was investigated also in vivo by injecting mice lacking PI3Kα in their hepatocytes and control mice with a low dose of the PI3Kβ-selective inhibitor TGX221. TGX221 had no effect on blood glucose levels of control mice but caused a severe hyperglycemic response in mice lacking PI3Kα in their liver (Molinaro et al. 2019). Overall it can be concluded that insulin-driven PI3K/AKT

signaling in the hepatocyte and insulin action in endogenous glucose production depend on redundant PI3Kα and PI3Kβ activities (Fig. 3b) (Molinaro et al. 2019).

7.4 PI3Kδ Promotes Lymphocyte-Driven Inflammation in Obesity

One study reported that the insulin-induced association of PI3K activity to IRS1 measured in vitro in liver extracts from mice lacking PI3Kα, can be largely inhibited using a PI3Kδ-selective inhibitor (Chattopadhyay et al. 2011). However, PI3Kδ protein is virtually undetectable in the hepatocyte by immunoblot, and PI3Kδ inhibition does not affect insulin-driven AKT phosphorylation in cultured primary hepatocytes lacking PI3Kα (Molinaro et al. 2019). Furthermore, PI3Kδ protein is far more abundant in Kupffer cells than in hepatocytes (Becattini et al. 2021). Hence, it is likely that most of the IRS1-associated PI3Kδ activity measured in vitro from liver extracts of mice lacking PI3Kα (Chattopadhyay et al. 2011) derives from Kupffer cells rather than the hepatocyte. Overall, to our knowledge, there is currently no evidence indicating that PI3Kδ plays a significant role in insulin-driven PI3K/AKT signaling within the insulin target cells mediating systemic glucose or lipid metabolism. However, a study has found that PI3Kδ/AKT signaling plays an important role in promoting the development of pro-inflammatory effector memory $CD4^+$ T cells in obesity and their migration to non-lymphoid tissues, such as the adipose tissue (Mauro et al. 2017). Thus, the authors speculate that this pathway may significantly contribute to metabolic inflammation and metabolic stress associated with obesity (Mauro et al. 2017). Future studies are required to define the role of PI3Kδ in obesity-related diseases.

7.5 PI3Kγ Promotes Adiposity, Metabolic Inflammation, and Insulin Resistance

Two independent studies have found that mice with systemic ablation of PI3Kγ (PI3Kγ-KO) are protected from diet-induced obesity, fatty liver, metabolic inflammation, and insulin resistance (Becattini et al. 2011; Kobayashi et al. 2011). One study proposed that PI3Kγ activity in leukocytes drives metabolic inflammation and, as a consequence, insulin resistance, fatty liver, and adiposity (Kobayashi et al. 2011). Using bone marrow transplantation, the second study found that loss of PI3Kγ in the non-hematopoietic compartment causes a leaner phenotype (Becattini et al. 2011). Reduced adiposity was closely associated with improved insulin sensitivity in these mice, indicating that reduced inflammation and improved insulin sensitivity in PI3Kγ-KO mice are likely consequent to their leaner phenotype (Becattini et al. 2011). Ablation of PI3Kγ protected mice from diet-induced obesity but did not

reduce adiposity in mice lacking functional leptin signaling (ob/ob mice and db/db mice), indicating that the effects of PI3Kγ ablation on reduced adiposity may depend on leptin (Breasson et al. 2017, 2018). PI3Kγ-KO mice showed increased phosphorylation of hormone-sensitive lipase (HSL) by PKA and exacerbated diet-induced thermogenesis, indicating that PI3Kγ may be a key regulator of catecholamine-driven lipolysis and thermogenesis (Becattini et al. 2011). Conditional ablation of PI3Kγ in hematopoietic and endothelial cells showed that the leaner phenotype observed in PI3Kγ-KO mice kept on high-fat diet, and most of their improved insulin sensitivity was not due to PI3Kγ activity in leukocytes (Breasson et al. 2017). However, loss of PI3Kγ activity in leukocytes caused a partial and transient improvement in insulin sensitivity associated with a reduced number of adipose tissue neutrophils and reduced expression of inflammatory genes (Breasson et al. 2017). These protective effects of PI3Kγ ablation in diet-induced obesity and insulin resistance were reproduced by a third independent laboratory (Torres et al. 2019). Furthermore, an additional independent study reported that mice lacking a catalytically active PI3Kβ and PI3Kγ display reduced adipose tissue mass, increased lipolysis, and browning of white adipose tissue (Perino et al. 2014).

Overall, PI3Kγ ablation protects mice from diet-induced obesity, promotes adipose tissue lipolysis, and thermogenesis by a leptin-dependent mechanism.

The specific cell type responsible for PI3Kγ action in the control of adiposity and the detailed molecular mechanisms for this action is unresolved.

8 Closing Remarks

Hyperglycemia is a substantial adverse effect of pan-PI3K inhibition, and compensatory hyperinsulinemia causes resistance to PI3K/AKT-targeted therapies (Hopkins et al. 2018; Janku et al. 2018) (Fig. 2b). This obstacle may be circumvented by selectively inhibiting catalytic PI3K subunits implicated in a specific pathological process but not essential for systemic metabolic homeostasis.

Therapies targeting PI3K/AKT signaling in hematopoietic cells may be particularly amenable to success since PI3Kδ and PI3Kγ proteins, which are most abundant in leukocytes, may be dispensable for insulin action in systemic metabolic homeostasis (Molinaro et al. 2019). Indeed, out of five PI3K inhibitors approved for clinical use, four are used to treat hematological malignancies. Nonetheless, because of the redundant role of PI3Kα and PI3Kβ in insulin signaling, inhibitors discriminating between PI3Kα and PI3Kβ may prove successful in treating solid tumors (Molinaro et al. 2019). An outstanding example is the approval of the PI3Kα-selective inhibitor Alpelisib for the treatment of breast cancer (Markham 2019).

Furthermore, the action of the PI3K/AKT pathway in metabolic homeostasis goes beyond its role in insulin signaling. To which extent the PI3K/AKT activity in anabolic metabolism applies to signals other than insulin is an interesting question, which is highly relevant to the field of immunometabolism.

The possible combinations of different PI3K regulatory subunits with different catalytic subunits and their modality of regulation generate substantial complexity and diversity by which the PI3K/AKT signaling can be recruited. Embracing this complexity and resolving the functional specificities and redundancies of different PI3K isoforms in metabolic homeostasis and diseases may pave the road to new therapies.

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The Role of PI3K Isoforms in Autoimmune Disease

Stephen G. Ward

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Abstract Aberrant overactivation of the immune system can give rise to chronic and persistent self-attack, culminating in autoimmune disease. This is currently managed therapeutically using potent immunosuppressive and anti-inflammatory drugs. Class I phosphoinositide-3-kinases (PI3Ks) have been identified as ideal therapeutic targets for autoimmune diseases given their wide-ranging roles in immunological processes. Although progress has been hampered by issues such as poor drug tolerance and drug resistance, several PI3K inhibitors have now received regulatory approval with many others in development, including several intended to suppress the immune response in autoimmune and inflammatory diseases. This chapter reviews the evidence for contribution of aberrant PI3K activity to a range of autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and type I diabetes) and possible therapeutic application of isoform-specific PI3K inhibitors as immunosuppressive drugs.

Abbreviations

APDS Activated PI3Kδ Syndrome

S. G. Ward (✉)
Department of Pharmacy and Pharmacology and Bath Centre for Therapeutic Innovation, University of Bath, Claverton Down, Bath B2 7AY, UK
e-mail: S.G.Ward@bath.ac.uk

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CNS	Central nervous system
CIA	Collagen-induced arthritis (CIA)
fMLP	N-formylmethionyl-leucyl-phenylalanine
(h)TNFα	Human tumour necrosis factor-α
MMPS	Matrix metalloproteinases
MS	Multiple sclerosis
mTORC2	MTOR complex 2
NOD	Non-obese diabetic
PDK-1	Phosphoinositide-dependent kinase-1
PH	pleckstrin homology
PI3K	phosphoinositide-3-kinase
PIP2	Phosphatidylinositol-(4,5)-bisphosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisphosphate
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus

1 Introduction

Phosphoinositide-3-kinases (PI3Ks) are lipid kinases that phosphorylate the 3-hydroxyl position of the inositol ring of phosphatidylinositol substrates within the plasma membrane and intracellular compartments. The class I PI3Ks include four isoforms (α, β, γ, and δ) that phosphorylate phosphatidylinositol-(4,5)-bisphosphate (PIP2) to produce phosphatidylinositol-(3,4,5)-trisphosphate (PIP3), an important second messenger molecule that regulates multiple cellular signalling pathways (Foster et al. 2012; Vanhaesebroeck et al. 2021). PI3Kα and PI3Kβ are broadly expressed, while PI3Kγ and PI3Kδ are mainly expressed in leukocytes. PI3Kδ is activated by tyrosine kinases (e.g. downstream of B cell and T cell receptors), G protein-coupled receptors and Ras superfamily of small GTPases (Foster et al. 2012; Vanhaesebroeck et al. 2021). Enhanced PI3K activity has been implicated in various cancers and inflammatory diseases and the pharmaceutical industry has put immense effort into developing PI3K inhibitors. For example, the oral selective PI3Kδ inhibitor idelalisib is approved for treatment of relapsed or refractory chronic lymphocytic leukaemia, small lymphocytic lymphoma and follicular non-Hodgkin lymphoma (Foster et al. 2012; Vanhaesebroeck et al. 2021; Furman et al. 2014; Gopal et al. 2014). Several other pan-isoform and isoform-specific inhibitors are in various stages of development (Foster et al. 2012; Vanhaesebroeck et al. 2021). This chapter considers the contribution of PI3Ks to the normal immune response and how dysregulation of this pathway can contribute not only to inflammatory but also to autoimmune diseases. PI3K activation occurs in response to a diverse array of receptors that are expressed on leukocytes and are responsible for both innate and adaptive immune responses as well as those that constitute a link between these two arms of the immune response, including mast cells and eosinophils. PI3Ks are activated by

antigen receptors, costimulatory receptors, Fc receptors, adhesion molecules, Toll-like receptors (TLRs) and cytokine receptors, as well as receptors for a variety of chemoattractants, including C5a, *N*-formylmethionyl-leucyl-phenylalanine (fMLP), chemokines and sphingosine-1-phosphate (Foster et al. 2012; Blunt and Ward 2012; Ball et al. 2014; Vanhaesebroeck et al. 2021).

Given their predominant expression in leukocytes, the role of PI3Kγ and PI3Kδ in various immune pathologies has been extensively explored in animal models. Mice in which the genes encoding PI3Kγ or PI3Kδ have been either ablated or altered to encode kinase-inactive mutants are viable, fertile and apparently healthy. However, when their immune system is challenged, they exhibit severely altered phenotypes demonstrating that PI3Kγ and PI3Kδ have nonredundant functions in leukocytes and that the activities of these isoforms in immune cells are crucial during chronic inflammatory diseases (Foster et al. 2012; Blunt and Ward 2012; Ball et al. 2014). Often these roles are distinct, requiring coordinated function of both isoforms at discrete steps of immune cell activation. PI3Kδ is functionally dominant in lymphocytes whereas PI3Kγ plays a more important role in myeloid cells, although this distinction is not absolute (Hawkins and Stephens 2015).

Although PI3K-dependent signalling has been identified as crucial for immune cell function, aberrant overactive PI3K signalling is known to result in immune-related pathologies (Blunt and Ward 2012; Ball et al. 2014). It is important to note that patients with deletion or loss-of-function mutations in *PIK3CD* demonstrate a serious block in B cell development as well as a range of immune dysregulatory diseases including inflammatory bowel disease, autoimmune hepatitis and juvenile idiopathic arthritis as well as susceptibility to a range of infections (Lucas et al. 2014; Sharfe et al. 2018; Sogkas et al. 2019; Swan et al. 2019). A gain-of-function E1021K mutation in the *PIK3CD* gene that encodes the PI3Kδ catalytic subunit, leads to Activated PI3Kδ Syndrome (APDS, chapter 8) (Angulo et al. 2013; Lucas et al. 2014; Stark et al. 2018). Immune-related defects in APDS patients include sinopulmonary infections and lymphadenopathy as well as increased likelihood of developing autoimmune and inflammatory complications with high frequency of autoantibodies, autoimmune cytopenias, arthritis and glomerulonephritis (Coulter et al. 2017; Lucas et al. 2016; Vanhaesebroeck et al. 2021).

2 Rheumatoid Arthritis

RA is a chronic autoimmune disease that predominantly causes inflammation in small joints of the hands and feet causing immobilisation and disability. In RA, the structure of the synovium is transformed into a pannus-like tissue that invades cartilage and erodes bone. Strong granulocyte and lymphocyte recruitment cause the inflamed synovium to consist of multiple inflammatory cells, such as macrophages, neutrophils and T and B cells. Hyperplasia of synovial lining cells (predominantly fibroblast-like synoviocytes) (Firestein 2003) occurs and damage to tissue is driven by aggressive inflammatory cytokine signalling in these joints. Given that PI3Kγ has

a pivotal role in mediating leukocyte migration and activation as well as mast cell degranulation (Foster et al. 2012), it was predicted that blocking PI3Kγ might be an effective strategy to fight RA. The collagen-induced arthritis (CIA) model reflects the immunological components of the disease and constitutes an acute T cell-mediated autoimmune arthritis model that focuses on the effector phase of arthritis. In this model, PI3Kγ null mice exhibited reduced paw swelling, synovial inflammation and cartilage erosion while inhibition of PI3Kγ decreased neutrophil infiltration as well as Th17 differentiation, a pro-inflammatory helper T-cell type characterised by expression of the cytokine interleukin (IL)-17 (Bergamini et al. 2012; Camps et al. 2005). However, while the dual PI3Kγ/δ inhibitor IP-145 could significantly reduce ankle swelling in a rat CIA model (Winkler et al. 2013), it was ineffective in phase 2 clinical trials (Vanhaesebroeck et al. 2021), highlighting that animal models do not always predict clinical outcomes in patients.

The transgenic overexpression of human (h)TNFα in mice leads to a chronic inflammatory destructive polyarthritis. This is similar to that observed in human RA and is ameliorated by treatment with neutralising anti-TNFα antibodies (Keffer et al. 1991). Loss of PI3Kγ in hTNFα transgenic mice also led to reduced arthritis in comparison to wild-type mice (Hayer et al. 2009). In contrast to the CIA model, PI3Kγ deficiency in hTNFα mice does not alter the recruitment of inflammatory leukocytes. However, PI3Kγ deficiency in hTNFα mice significantly reduces cartilage damage through reduced expression of matrix metalloproteinases (MMPs) in fibroblasts and chondrocytes. PI3Kγ expression is significantly higher in the synovium of RA patients compared with synovium from osteoarthritis patients. Furthermore, inhibition of PI3Kγ reduced TNFα-induced MMP production in fibroblasts isolated from human RA patients (Hayer et al. 2009). These studies, therefore, highlight that the mechanistic role of PI3Kγ in RA extends beyond its established role in mediating immune cell migration.

PI3Kδ mRNA and protein expression are also higher in RA than osteoarthritis synovium (Bartok et al. 2012) and PI3Kδ mRNA can be induced in cultured synoviocytes by inflammatory cytokines. In a K/BxN-serum transfer model of arthritis, in which neutrophils and leukotriene B4 (LTB4) participate in the effector phase of inflammatory arthritis, selective inhibition of PI3Kδ diminishes joint erosion to a level comparable to inhibition of its PI3Kγ counterpart. Induction and progression of joint destruction were significantly reduced in the absence of both PI3K isoforms (Randis et al. 2008). The K/BxN mouse model also revealed reduced disease development in PI3Kβ deficient mice at low, but not high doses of serum transfer, while additional PI3Kδ deficiency markedly reduced disease severity at high serum transfer doses, indicating a role for dual PI3Kδ/PI3Kβ inhibitors in this context (Kulkarni et al. 2011).

Important differences between PI3Kγ and PI3Kδ have been noted in the mechanisms underpinning joint destruction. For example, in a model of osteoclastogenesis, the PI3Kδ-selective inhibitor IC87114 significantly inhibited the generation of osteoclasts, whereas selective inhibition of PI3Kγ with AS605240 had no effect (Toyama et al. 2010). Taken together, these lines of evidence suggest that dual inhibition

of PI3Kγ and PI3Kδ would be more therapeutically beneficial than targeting one isoform alone in this group of diseases.

3 Systemic Lupus Erythematous

SLE is a complex multiorgan autoimmune disease characterised by the presence of autoreactive antibodies and chronic activation of the immune system. Typically, initial overproduction of memory $CD4^+$ T lymphocytes leads to hyperactivity of polyclonal B lymphocytes and induces their rapid expansion and production of autoantibodies. The presence of antinuclear autoantibodies can lead to the formation of complexes that are retained in the kidney in 50% of all patients and can lead to leukocyte infiltration, ultimately causing renal failure and glomerulonephritis (Kaul et al. 2016). Current therapies poorly control disease indicators, necessitating potent immunosuppression to achieve remission.

In silico analysis of drug targets based on gene signatures revealed the PI3K pathway as a promising target in SLE (Toro-Dominguez et al. 2017). Moreover, increased PI3K activity positively correlates with BAFF levels, a cytokine involved in SLE pathogenesis (Ge et al. 2017). PI3Kδ enhances the BAFF-mediated cellular survival and maturation of B cells (Henley et al. 2008). PI3Kδ (but not PI3Kγ) activity is significantly increased in T cells of SLE patients compared to normal individuals, and this difference was greatest in patients with active disease (Suarez-Fueyo et al. 2011). The reason for this increased PI3Kδ activity in SLE patients is unclear but correlated with resistance of activated and memory T cells to activation-induced cell death and an increased number of memory T cells (Suarez-Fueyo et al. 2011). Moreover, in mouse models, genetic or pharmacological inhibition of PI3Kδ signalling has been shown to reduce the incidence and severity of SLE features (Haselmayer et al. 2014; Maxwell et al. 2012; Suarez-Fueyo et al. 2014; Wang et al. 2014). Hence, PI3Kδ inhibitors potentially may be effective for SLE treatment or prevention, regardless of the nature of the factor that triggered PI3Kδ activation initially**.** However, given that SLE is a highly heterogeneous disease, it is likely that PI3Kδ is activated only in a subgroup of the patients. Indeed, there are reports that a small (20%) subgroup of SLE patients exhibit biomarkers associated with ARDS, such as increased numbers of transitional B cells and antinuclear autoantibodies (Li et al. 2019; Sim et al. 2005; Simon et al. 2016). Thus, treating all SLE patients with PI3Kδ inhibitors would be either unnecessary or potentially damaging and would also be difficult to assess in clinical trials, where beneficial effects in some patients will be masked by the lack of the effect in others. Hence, treatment with PI3Kδ inhibitors has to be personalised only to those patients who have increased PI3Kδ activity and will require robust reproducible biomarkers of increased PI3Kδ activity in SLE patients.

Several lines of evidence have also suggested dysregulated PI3Kγ-dependent signalling in mouse models of SLE. For example, deletion of PI3Kγ in a mouse

model reduced the survival of pathogenic memory $CD4^+$ lymphocytes, which ultimately led to inhibition of glomerulonephritis and enhanced survival rate (Barber et al. 2006). Furthermore, treatment of these mice with the PI3Kγ inhibitor AS605240 also reduced autoantibody production and increased survival (Barber et al. 2005). Finally, IPI-145 is a potent, oral class I PI3K inhibitor targeting PI3Kδ and PI3Kγ that showed potent activity in a rodent SLE model (Winkler et al. 2013). This illustrates the potential therapeutic value of combined PI3Kδ and PI3Kγ blockade.

4 Multiple Sclerosis

MS is an autoimmune disease characterised by build-up of scar tissue (sclerosis) in the brain and/or spinal cord. It is caused by immune cell-mediated destruction of the myelin sheath surrounding nerve fibres. This leads to impaired signals to and from the brain affecting muscle control, vision, balance and causing fatigue, loss of sensation or numbness. Experimental autoimmune encephalomyelitis (EAE) is an induced method of autoimmune inflammation of the central nervous system (CNS) commonly used to model MS in rodents. Substantial evidence now indicates that the T cell lineage most likely to be driving EAE pathogenesis is Th17 cells (Kleinschek et al. 2007). Genetic targeting of PI3Kγ in a Th17-driven EAE model delayed progression of motor dysfunction, reduced pro-inflammatory chemokines and decreased numbers of immune cells that infiltrated the meninges of PI3Kγ-deficient mice (Rodrigues et al. 2010). Genetic and pharmacological targeting of PI3Kγ indicated that it plays an important part in mediating leukocyte survival rather than leukocyte adhesion in this experimental model of MS (Berod et al. 2011; Li et al. 2013).

Signalling through PI3Kδ has been also reported to be involved in the pathogenesis of EAE. In this regard, T cell activation and function during EAE were markedly reduced and fewer T cells were observed in the CNS of mice transgenically expressing inactivated PI3Kδ (Haylock-Jacobs et al. 2011). Similar to observations made in the PI3Kγ-deficient mice, the proportion of T cells undergoing apoptosis at early stages of EAE was increased in the absence of PI3Kδ activity. Furthermore, there was a significant defect in Th17 cellular responses during EAE in the absence of PI3Kδ activity (Haylock-Jacobs et al. 2011), while the PI3Kδ inhibitor IC87114 inhibited Th17 cell generation in vitro (Haylock-Jacobs et al. 2011).

Collectively, these data indicate that both PI3Kγ and PI3Kδ contribute to the pathogenesis of EAE, influencing cell survival, differentiation and migration mechanisms. Thus, dual targeting of PI3Kγ and PI3Kδ might be a therapeutic option for the treatment of MS. Under homeostatic conditions, PI3Kδ is required to maintain normal Treg cell development, but loss of PI3Kδ specifically in Treg cells does not lead to autoimmunity. It is interesting to note, however, that combined loss of PI3Kα and PI3Kδ, resulted in increased EAE disease severity. Moreover, mice lacking

PI3Kα and PI3Kδ in Treg cells developed spontaneous peripheral nerve inflammation. Together, these results show a key role for several PI3K isoforms in Treg cell-mediated protection against CNS inflammation (Stark et al. 2020).

5 Type 1 Diabetes

Type 1 diabetes (T1D) is an autoimmune disease where activated T lymphocytes infiltrate the islets of Langerhans causing local inflammation of the pancreas leading to β-cell dysfunction and death (Cantor and Haskins 2007. In pre-clinical studies using the non-obese diabetic (NOD) mouse model of T1D, the specific PI3Kγ inhibitor AS605240 revealed that PI3Kγ is important in regulating the balance of T lymphocyte subsets during the pathogenesis of T1D (Azzi et al. 2012). In this regard, AS605240 suppressed autoreactive T lymphocytes and T lymphocyte infiltration into pancreatic islets while promoting expansion of immunosuppressive regulatory T lymphocytes. In addition, PI3Kγ inhibition not only prevented the development of diabetes in prediabetic NOD mice but could reverse established hyperglycaemia in NOD mice (Azzi et al. 2012).

The PI3Kδ isoform is known to play important roles in B lymphocyte biology, including activation, proliferation and antigen presentation, all of which can contribute to pathogenic inflammatory processes. Indeed, NOD mice that lack B cells do not develop spontaneous autoimmune diabetes seen in wild-type NOD mice (Serrese et al. 1996). Interestingly, in the NOD mouse model, the PI3Kδ inhibitor IC87114 delayed the onset, reduced severity and prevented progression of autoimmune diabetes in this mouse model and reduced infiltration of leukocytes into the pancreatic islets (Durand et al. 2013). Furthermore, in vitro activation of diabetogenic T lymphocytes by NOD B cells was also inhibited by IC87114. Hence, the PI3K pathway is a key driver of T and B lymphocyte activation and expansion in T1D, making it an attractive target for therapeutic intervention.

6 Concluding Remarks

The γ and δ isoforms of PI3K have important nonredundant roles in multiple cells of the immune system. Consequently, alterations of the PI3K signalling pathway can lead to inflammatory and autoimmune disorders as well as leukaemia. This, together with the continued design and development of improved PI3K inhibitors offers opportunities to manipulate the PI3K signalling network in immune cells, not only for autoimmune diseases but also for inflammatory conditions and transplantation as well as cancer. The latter could include targeting non-leukaemia cancers, given the upregulation of PI3Kγ and PI3Kδ in some forms of non-immune cell cancers, although one concern would be potential impairment of the immune system that may impair the endogenous antitumour response.

Although there is optimism for targeting PI3K in autoimmune disease, there remain key questions surrounding this therapeutic approach. Firstly, PI3K signalling comprises many areas of functional redundancy and plasticity, so targeted therapies are unlikely to have an effect across the range of conditions in which PI3K is involved. Second, targeting of PI3K catalytic isoforms does not necessarily silence PI3K signalling because AKT can be activated independently of the recognised phosphoinositide-dependent kinase-1 (PDK1)/mTORC2/PH-domain-mediated mechanisms (Guo et al. 2011). Thirdly, resistance mechanisms to PI3K-targeted therapy have been reported (Ilic et al. 2011). Finally, as already mentioned for SLE, many autoimmune diseases are multi-factorial in their pathogenesis. Hence, for any individual autoimmune condition, not all patients might benefit from a particular PI3K isoform-specific inhibitor. This emphasises the need for accurate biomarkers that can inform and direct precise and personalised medicine. Treatment with PI3K inhibitors should be personalised only to those patients who have increased PI3K activity and this will require robust reproducible biomarkers of increased PI3K isoform(s) activity in autoimmune patients.

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AKT Isoforms in the Immune Response in Cancer

Zayd Ahmad and Payaningal R. Somanath

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Abstract AKT is a protein kinase that exists in three isoforms: AKT1, AKT2, and AKT3. Though similar in structure, these isoforms display different effects. AKT is activated downstream of PI3K, and together, this signaling pathway helps regulate cellular processes including cell growth, proliferation, metabolism, survival, and apoptosis. Disruption in these pathways has been associated with disorders including cardiovascular diseases, developmental disorders, inflammatory responses, autoimmune diseases, neurologic disorders, type 2 diabetes, and several cancers. In cancer, deregulation in the PI3K/AKT pathway can be manifested as tumorigenesis, pathological angiogenesis, and metastasis. Increased activity has been correlated with tumor progression and resistance to cancer treatments. Recent studies have suggested that inhibition of the PI3K/AKT pathway plays a significant role in the development, expansion, and proliferation of cells of the immune system. Additionally, AKT has been found to play an important role in differentiating regulatory T cells, activating B cells, and augmenting tumor immunosurveillance. This emphasizes AKT as a potential target for inhibition in cancer therapy. This chapter reviews AKT structure and regulation, its different isoforms, its role in immune cells, and its modulation in oncotherapy.

Z. Ahmad · P. R. Somanath (✉)
Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia and Charlie Norwood VA Medical Center, Augusta, GA 30912, USA
e-mail: sshenoy@augusta.edu

P. R. Somanath
Georgia Cancer Center, Vascular Biology Center and Department of Medicine, Augusta University, Augusta, GA 30912, USA

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1 Introduction

Over thirty years ago, AKT was discovered and identified as an oncogene in the transforming retrovirus AKT8 (Coffer and Woodgett 1991). Due to its similarity in amino acid sequence to protein kinase A and protein kinase C, it is also known as protein kinase B. Since its discovery, multiple studies have demonstrated its role in cell survival, proliferation, and growth (Staal 1987). Present in nearly every cell in the body, it has even further been associated with resistance to apoptosis, cellular metabolism, insulin sensitivity, angiogenesis, and cellular migration (Datta et al. 1999). AKT is now understood to be an effector of multiple upstream regulatory inputs, most notably phosphoinositide-3-kinase (PI3K). A vast majority of growth factor responses in endothelial cells, epithelial cancer cells, inflammatory cells, etc. are mediated by this pathway (Burgering and Coffer 1995; Stoeltzing et al. 2006; Han et al. 2012; Kim et al. 2008). PI3K activation itself occurs downstream of multiple receptors involving G-coupled protein receptors, tyrosine kinase receptors, cytokine and costimulatory signaling, and others (Cantley 2002; Gonzalez and McGraw 2009; Buzzi et al. 2010; Chen et al. 2010; Grabinski et al. 2011; Okano et al. 2000). PI3K is responsible for the production of phosphatidylinositol-(3,4,5)-triphosphate ($PI_{3,4,5}P3$, PIP3), which then recruits downstream effectors containing the pleckstrin homology (PH) domain, including AKT (Cantley 2002).

In the immune system, it is thought that the PI3K/AKT pathway plays a significant role in the function and development of a variety of cells such as neutrophils, macrophages, dendritic cells, and T cells (Damoulakis et al. 2014; Kulkarni, et al. 2011; Battaglia et al. 2005; Crellin et al. 2007; Marshall et al. 2012; Rodriguez-Borlado et al. 2003). Inhibition of this pathway has been associated with increased expression of Forkhead box protein P3 (FoxP3) in regulatory T cells (Tregs). Treg cells have a crucial function in the prevention of autoimmunity and the resolution of inflammation (Sakaguchi et al. 2010). In cancer cells, deregulation of AKT has long been recognized to pave the road for oncogenic transformation and tumor growth (Martini et al. 2014). However, additional studies have also implicated the PI3K/AKT pathway, and its role in the proliferation and expansion of Tregs, in maintaining the immunosuppressive tumor microenvironment (Fruman and Rommel 2014). This suggests that while cancer cells depend on AKT expression for proliferation and tumorigenesis, inhibition of the PI3K/AKT/mTOR pathway may be the mechanism by which cancer cells avoid destruction by the immune system.

The functional diversity of AKT can be attributed to its diverse set of substrates. AKT is responsible for regulating the activation, and more commonly, the deactivation of numerous downstream signaling nodes such as glycogen synthase 3β (GSK3β), mammalian target of rapamycin complex 1 (mTORC1), and Forkhead box protein O1 (FoxO1) (Manning and Toker 2017; Somanath et al. 2006). Dysfunction or deregulation of AKT activity has been identified in cardiovascular diseases, developmental disorders, inflammatory responses, autoimmune diseases, neurologic disorders, type 2 diabetes, and several cancers. These processes have been reviewed

elsewhere (Manning and Toker 2017). In cancer, AKT deregulation has been associated with tumorigenesis, angiogenesis, and metastasis (Brazil et al. 2004). Unregulated AKT activation through mutations is commonly seen in multiple cancer types. In fact, over 50% of human tumors display hyperactivation of AKT. AKT hyperexpression has been also observed in breast, ovarian, prostate, lung, and gastric cancers, as well as melanoma, and lymphoma (Song et al. 2019; Alwhaibi et al. 2019; Qiao et al. 2007; Shan et al. 2017; Kim et al. 2017; Horn et al. 2017; Wang et al. 2017a; Wei et al. 2019). Because of this, the PI3K/AKT/mTOR pathway has been identified as a major target for the treatment of malignancy, with targeted AKT inhibition being proposed as a therapeutic route (Wang et al. 2017b). However, due to the diverse functions attributed to its wide array of substrates and upstream regulators, broadly inhibiting this pathway to treat cancer has proven complicated. Furthermore, AKT exists in three different isoforms: AKT1, AKT2, and AKT3. These isoforms were traditionally thought to be functionally redundant, and much of the published literature did not make distinctions between them (Santi et al. 2010). With the development of isoform-specific antibodies, isoform-specific small interfering RNA, and gene knockout or overexpressing mice, various individual functions of each AKT isoform have been demonstrated in many cell types in various physiological and pathological environments (Buzzi et al. 2010; Chen et al. 2010; Grabinski et al. 2011; Okano et al. 2000; Santi and Lee 2010; Sanidas et al. 2014; Tang et al. 2014; Yu et al. 2015; Kitz et al. 2016). However, the functions and specificities of their signaling cascades are still relatively poorly understood and need further investigation (Santi et al. 2010).

2 AKT Structure and Regulation

AKT is a serine-threonine kinase that is expressed ubiquitously in most cells (Stoeltzing et al. 2006). AKT exists in three isoforms: AKT1, 2, and 3 (Bellacosa et al. 2004). Despite being encoded in different genes, they share greater than 80% homology in their structure and substrate specificities (Zhang et al. 2006). Common characteristics between the isoforms include an N-terminal PH domain, a catalytic domain with a threonine residue, and a C-terminal regulatory domain with a serine residue (Kumar and Madison 2005). Activation of AKT is initiated by either receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs) that translocate class I PI3K to the plasma membrane. PI3K is known to phosphorylate $PI_{4,5}$ P2 to generate PIP3. PIP3 then binds to the PH domain of AKT, translocating it to the plasma membrane (Franke et al. 1995). Phosphoinositide-dependent-kinase-1 (PDK1) phosphorylates AKT at the threonine 308 site (Alessi et al. 1997). mTORC2 phosphorylates AKT at the serine 473 residue and phosphorylation of both sites is required for full activation (Sarbassov et al. 2005). Full activation of AKT is crucial for the activation of key downstream targets such as mTORC1, glycogen synthase kinase-3 (GSK3), FoxO1, and FoxO3a (Jacinto et al. 2006). mTORC1 and FoxO proteins have great relevance in the development and function of regulatory T cells (Jacinto et al. 2006; Delgoffe et al. 2009; Haxhinasto et al. 2008; Kerdiles et al. 2010).

Dephosphorylation or inactivation of the threonine residue is conducted by protein phosphatase 2 (PP2) (Andjelkovic et al. 1996). Deactivation of the serine residue is also mediated by PH domain and leucine rich repeat protein phosphatases (PHLPP) (Brognard et al. 2007). The tumor suppressor gene, PTEN, antagonizes the activation of AKT by reverting PIP3 to PIP2 (Maehama and Dixon 1998). Genetic mutations that result in the loss, reduced expression, or impaired function of PTEN or PHLPP have been found in many cancers (Chalhoub and Baker 2009; Chen et al. 2011), and the AKT isoforms themselves are considered bona fide oncogenes (Manning and Toker 2017) (Fig. 1).

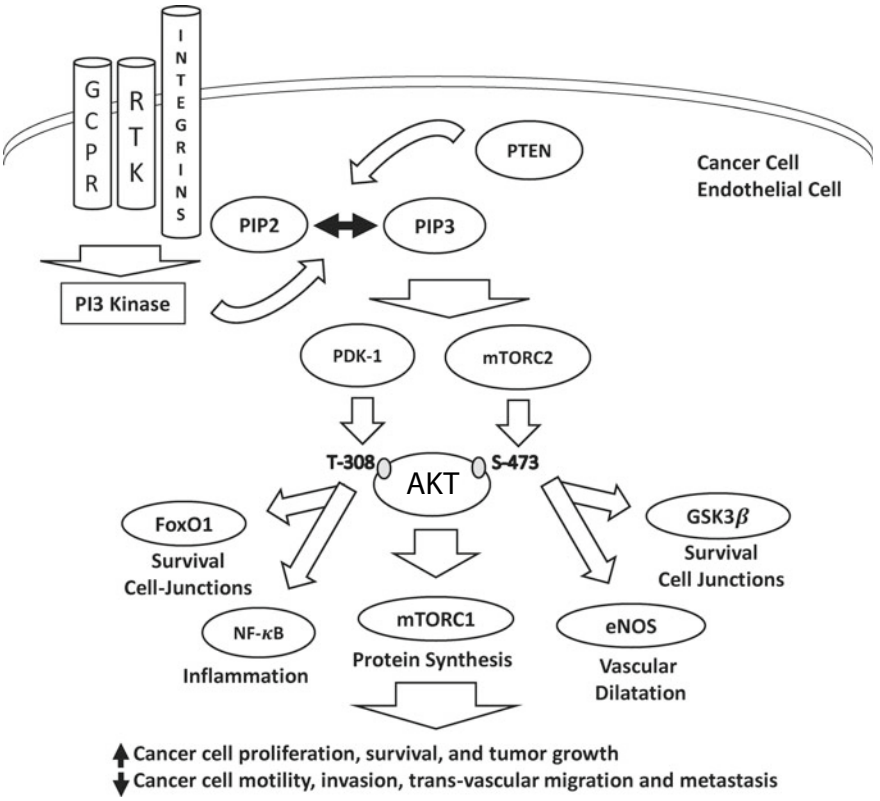

Fig. 1 Regulation of the AKT pathway in tumor and endothelial cells. The GPCRs, RTKs, and integrins activate PI3K to convert PIP2 into PIP3, in turn activating PDK1 and mTORC2 subsequently phosphorylating Thr 308 and Ser 473 of AKT, respectively. Once activated, AKT phosphorylates a plethora of substrates including but not limited to GSK3, mTORC1, NFκB, eNOS, and FoxO1, modulating cancer cell survival, proliferation, tumor growth, motility, invasion, trans-vascular migration, and metastasis

3 The Role of AKT in Immune Cells

The PI3K/AKT pathway has a distinguished role in glucose metabolism, cell growth, resistance to apoptosis, maturation, and proliferation of cells (Martini et al. 2014). The pathway is also involved in myelopoiesis, which occurs in response to a variety of growth factors (Dexter and Spooncer 1987; Ogawa 1993; Szabolcs et al. 1995). In T cells, the pathway is involved in the differentiation of $CD4^+CD127^-CD25^+FoxP3^+$ T regulatory cells (Tregs) (Fruman and Rommel 2014). Tregs express high levels of CD25 and CTLA4 (cytotoxic T-lymphocyte-associated protein or CD152) and have important functions in controlling inflammation and maintaining self-tolerance, thereby controlling autoimmunity (Sakaguchi et al. 2010). FoxP3 is the master regulator transcription factor in the development, maintenance, and function of Tregs, and it is in part regulated by the activity of AKT (Fontenot et al. 2003; Hori et al. 2003). While Tregs in homeostasis do not produce pro-inflammatory cytokines, in some autoimmune conditions and infectious diseases Tregs produce interleukin-17 (IL-17) and interferon-gamma (IFN-γ) (Dominguez-Villar et al. 2011; McClymont et al. 2011). The effects of AKT activation on the proliferation of Tregs may be dependent on the Treg location or stage of development (Fig. 2).

Studies examining the effect of PI3Kδ knockout in murine models found a resulting increased proportion of thymic Tregs (Okkenhaug et al. 2002; Herman et al. 1991). This is thought to be mediated by the effect of AKT suppression. AKT phosphorylates FoxO1, which is subsequently translocated from the nucleus to the cytoplasm (Klebanoff et al. 2016; Ouyang et al. 2012). Due to the reduced availability of FoxO1 in the nucleus, there is impaired binding to the FoxP3 promoter and other Treg-specific targets, impairing the Treg transcriptional program (Pompura and Dominguez-Villar 2018; Ouyang et al. 2010). T cells also require signals from the T cell receptor (TCR) or the IL-7 receptor to regulate survival, metabolism, and growth (Labrecque et al. 2001; Polic et al. 2001; Schluns et al. 2000; Tan et al. 2001). Signaling from the TCR and CD28, a costimulatory molecule, drive the growth, proliferation, and terminal differentiation of these cells (Alegre et al. 2001). This process causes the T cells to express glucose transporter 1 (Glut1), which allows the T cells to rapidly expand due to increased glucose uptake and glycolysis (Frauwirth et al. 2002). AKT is utilized in the signal transduction pathway of CD28 and without costimulation through this pathway, T cells fail to proliferate (Frauwirth et al. 2002).

In B cells, proliferation and maturation take place in the germinal center (GC). Located within secondary lymphoid tissues, antigen-activated B cells undergo expansion, class switching, rapid proliferation, and maturation in the GC (Cho et al. 2001). B cells are critical in the development of humoral immunity by the generation of antigen-specific antibodies. The PI3K/AKT pathway plays an important role in this process. AKT is responsible for B cell activation after phosphorylation by PDK1 (Baracho et al. 2014). In response to signals from T follicular helper cells, B cells undergo antibody class switching and affinity maturation. Mutations in FoxO1 have been observed in B cell lymphomas, including diffuse large B cell lymphoma and Burkitt's lymphoma. High expression of FoxO1 is associated with increased mortality

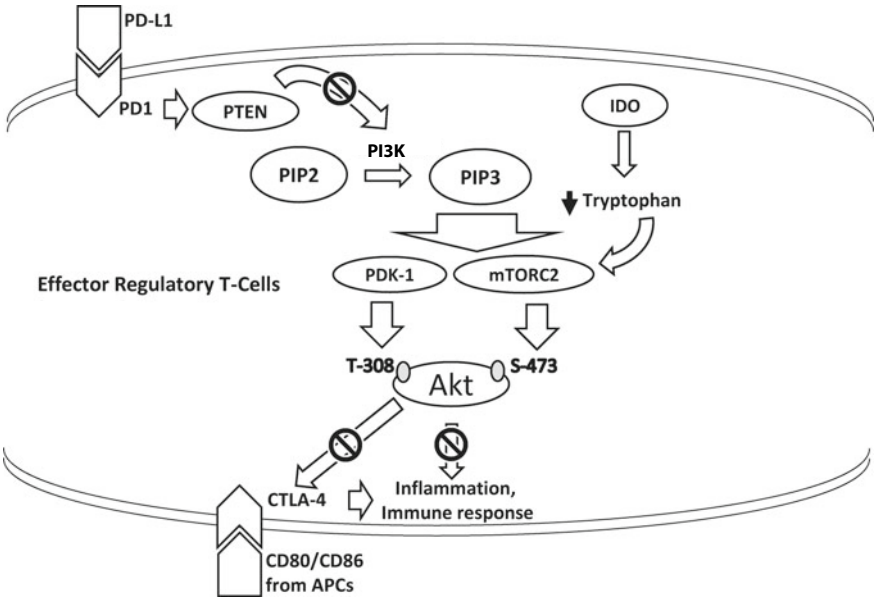

Fig. 2 The effects of AKT activity modulation in Tregs. In Tregs, the AKT pathway is maintained in a quiescent stage. Upon binding of PD-L1 to its receptor PD1 in Tregs, the activated PTEN, which is highly expressed on Tregs, inhibits the PI3K/AKT pathway, in turn, stabilizing activated Tregs to suppress inflammation and immune response. Activated AKT suppresses the expression of CTLA4 in Tregs that binds to the CD80/CD86 of antigen-presenting cells (APCs) leading to its de-differentiation into conventional T cells (Tconv) and promoting immune response and inflammation. Inhibition of AKT activity in Tregs will result in increased CTLA-4 expression, in turn, suppressing inflammation and immune response

in these patients (Trinh et al. 2013). Studies have also shown that there may be differences in the function of the individual AKT isoforms on the development of B cells as well (Zhu et al. 2019) (Fig. 3).

4 AKT1

AKT1 is the most highly expressed AKT isoform, being found in the majority of body tissues (Wang et al. 2017b). In patients with relapsing–remitting multiple sclerosis, AKT1 was markedly upregulated in Tregs which was associated with higher levels of IFNγ secretion by Tregs. Inhibition of AKT1 by either gene silencing or an AKT inhibitor led to a decrease in the expression of IFNγ and an increase in the suppressive capacity of Tregs (Kitz et al. 2016). AKT1 overexpression has been seen in 40% of breast and ovarian cancers and 50% of prostate cancers (Bellacosa et al. 2004; Cheng et al. 2005). Dysregulation of AKT1 is also often associated with leukemia and, expression of AKT1 is often seen in BCR/Abl leukemia and Philadelphia-positive

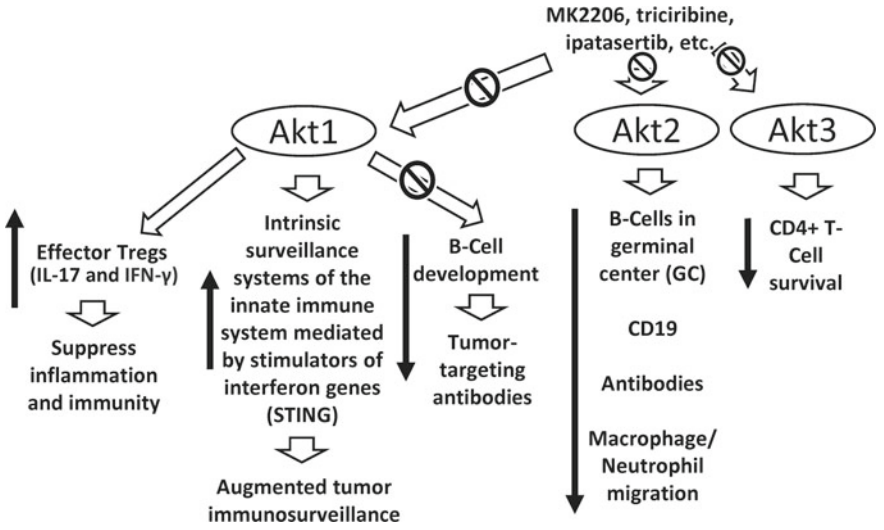

Fig. 3 Pharmacological inhibition of AKT isoforms for cancer therapy may have diverse effects on immune and inflammatory cells. Although pharmacological inhibition of AKT in the early stages of cancer may have beneficial effects on reducing the tumor burden by suppressing proliferation and inducing cancer apoptosis, its effects on immune cells are varied. Whereas AKT1 suppression by agents such as MK2206, triciribine, and ipatasertib increases Treg numbers to suppress inflammation and immune response, they promote immunosurveillance. Targeting AKT2 may suppress B cell activation and production of antitumor antibodies, and likely promote $CD4^+$ T cell apoptosis

myeloid cells (Skorski et al. 1995, 1997; Kochetkova et al. 1997). Another major function of AKT1 is to promote B cell proliferation and growth. In mouse models, AKT1/2 deficiency caused impaired GC response and decreased the generation of high-affinity, antigen-specific antibodies. These B cells also had reduced glucose and oxygen consumption and decreased mitochondrial mass. This was not observed in AKT1/3 deficient models (Zhu et al. 2019).

Recent studies have suggested that inhibition of the PI3K/AKT/mTOR pathway can augment tumor immunosurveillance. In tumor settings, DNA fragments are often released into the cytosol from the nucleus or mitochondria (Fu et al. 2015). Intrinsic surveillance systems of the innate immune system mediated by stimulators of interferon genes (STING) are involved in the detection of these DNA fragments. This ultimately leads to the production of interferons, increases cellular senescence, boosts antitumor immunity, and may even be a potential treatment for cancers (Fu et al. 2015; Dou et al. 2017). A recent study demonstrated that disruption of STING signaling by human epidermal growth factor receptor (HER2) coincides with the activation of AKT1 (Wu et al. 2019). HER2 expression is commonly associated with metastatic breast cancer and ovarian, prostate, and lung cancers (Moasser 2007; Arteaga and Engelman 2014). Interestingly, AKT2 and AKT3 were not recruited, suggesting disparities in the signaling capacities of the isoforms. HER2 induction and AKT1 recruitment in this setting were also associated with decreased $CD4^+$ and $CD8^+$

T cell migration into tumors (Wu et al. 2019). Pharmacologic targeting of AKT1 in this setting may increase the immune response to malignancies by modulating STING-mediated cytosolic DNA sensing of the innate immune system (Fig. 3).

5 AKT2

Although less extensively expressed than AKT1, AKT2 is found in the liver, skeletal muscle, and adipose tissue (Wang et al. 2017b). AKT2 overactivity has been observed in 30–40% of pancreatic and ovarian cancers (Bellacosa et al. 2004; Cheng et al. 2005). It is also associated with malignant tumor development in the liver, pancreas, and colon (Xu et al. 2004; Hers et al. 2011; Roy et al. 2002). *Akt2*$^{-/-}$ mice display decreased numbers of B cells in the GC and impaired B cell activation (Du et al. 2020). Inhibition of AKT2 causes decreased phosphorylation of CD19, which is an important co-receptor on the B cell surface (Engel et al. 1995). Impairment of CD19 phosphorylation results in severe deficiencies in humoral and antigen responses (Zelm et al. 2006). The deficiency in AKT2 has been also shown to correlate with defective migration of neutrophils and macrophages (Zhang et al. 2009). In addition, studies have demonstrated that AKT2 is necessary for the chemotaxis of macrophages (Stavrou et al. 2018).

6 AKT3

Less insights have been obtained from AKT3 compared to the other isoforms. AKT3 is primarily expressed in the brain and testes (Wang et al. 2017b). Over expression of the isoform has been noted in prostate and estrogen receptor-negative breast cancer (Altomare and Testa 2005; Bellacosa et al. 2005). Recent studies have also shown that AKT3 is highly expressed in invasive bladder carcinomas characterized by fibroblast growth factor 2 (FGF2) (McNiel and Tsichlis 2017). FGF2 bladder carcinoma correlates with epithelial to mesenchymal transition-promoting transcription factors and poor prognosis (Allen and Maher 1993; Nguyen et al. 1993; Inoue et al. 2000). FGF2 bladder carcinomas also had higher levels of CTLA4, PD1, and PD-L1, which may indicate immune blockade by checkpoint activation (McNiel and Tsichlis 2017). These immune checkpoints inhibit the host response and are expressed by tumors as a mechanism to evade immune recognition and maintain the tumor microenvironment (Topalian et al. 2012; Yao et al. 2013; Chen and Flies 2013).

Another study determined that targeting AKT3 using micro-RNAs (miRNA) can enhance $CD4^+$ T cell apoptosis by inhibiting proliferation and activation of $CD4^+$ T cells (Fig. 3) (Sang et al. 2016). miRNAs inhibit translation or promote degradation of targeted messenger RNA to cause changes in downstream regulatory targets (Lopez-Ramirez and Nicoli 2014; Osella et al. 2014). One specific miRNA, miR150, suppresses the immune response by downregulating genes involved in the maturation

of T cells, B cells, and natural killer cells (Zhou et al. 2007; Zheng et al. 2012). Levels of miR150 can be used as a predictive indicator for the occurrence of acute graft versus host disease (aGVHD) in patients who have received allogeneic hematopoietic stem cell transplant (allo-HSCT) (Ferrara et al. 2009; Sang et al. 2015). $CD4^+$ T cells from the donor in allo-HSCT are the main effector cells that cause aGVHD in these patients, along with cytokines such as IFNγ and IL-2 (Yu et al. 2011). Inhibition of AKT3 by miR150 may reduce the risk of aGVHD after allo-HSCT by inducing immune tolerance (Sang et al. 2016).

7 AKT Modulation in Oncotherapy

The PI3K/AKT pathway has a distinguished role in glucose metabolism, resistance to apoptosis, and proliferation (Martini et al. 2014). Deregulation of this pathway leads to oncogenic transformation and tumor growth (Somanath et al. 2009). It is well known that tumor cells thrive by avoiding detection by the immune system and maintaining the immune-suppressive tumor microenvironment (Sundararajan and Vogelzang 2015). Studies have now suggested that the PI3K/AKT pathway is involved in preserving that microenvironment (Fruman and Rommel 2014). AKT1 suppression is associated with an increased expression of FoxP3 on Tregs. These T cells are characterized by the expression of CD25, CD103, and CTLA4. They are also characterized by the low expression of CD127 are responsible for the resolution of inflammation (Sakaguchi et al. 2010; Liston and Gray 2014). Furthermore, T cells expressed in the tumor microenvironment have a high level of PTEN, PD1, and Indoleamine 2,3 dioxygenase (IDO) (Sharma et al. 2013, 2015). CTLA4, PD1, and PD-L1 are immune checkpoints that are responsible for augmenting T cell-mediated tumor response (Leach et al. 1996; Hurwitz et al. 1998; Agata et al. 1996; Keir et al. 2007).

PD1 is an immunoreceptor expressed on the surfaces of T cells and B cells and is a target for multiple approved cancer treatments (Kirkwood et al. 1996; Francisco et al. 2009; Patsoukis et al. 2012). PD1 inhibits the activation of AKT by preventing CD28-mediated PI3K activation (Parry et al. 2005). PD-L1 is the ligand for PD1 and their binding causes negative regulation of T cell activation and prevents anti-tumor effects (Latchman et al. 2001; Blank et al. 2005). IDO has also been found to prevent the activation of AKT through the mTOR pathway (Sharma et al. 2013). This suggests that while cancer cells depend upon AKT for proliferation, inhibition of AKT is the mechanism by which tumors avoid destruction by the immune system. PTEN inhibits AKT by dephosphorylating PIP3 and preventing translocation of the kinase for activation. PTEN expression in tumor cells correlates with increased immunosuppressive factors such as VEGF (Dong et al. 2014; Peng et al. 2016). VEGF drives immature dendritic cells, Tregs, and myeloid-derived suppressor cells to the tumor microenvironment to suppress the immune system (Voron et al. 2014). Blocking VEGF in murine models has been shown to increase the number of infiltrating antitumor T cells (Hers et al. 2011). Currently, immune checkpoint

inhibitors and VEGF inhibitors are monoclonal antibodies that are used to treat a wide variety of malignancies (Hodi et al. 2010; Motzer et al. 2015; Kwon et al. 2014; Gettinger et al. 2015). However, these targeted therapies, while showing positive initial results, often lead to relapse in many patients through various resistance mechanisms (O'Donnell et al. 2018).

Despite the efficacy of PD1 and CTLA4 inhibitors that inhibit deregulation of the PI3K/AKT pathway, new studies have suggested that inhibition of AKT may enhance the intrinsic antitumor properties of the immune system (Peng et al. 2016). Paradoxical of earlier studies, some new reports have shown that deletion of PTEN, and activation of AKT, can correlate with decreased T cell infiltration into the tumor sites. This leads to worse outcomes and resistance to PD1 inhibitors (Peng et al. 2016). This may be due to the activity of MDSCs (myeloid-derived suppressor cells), as the PI3K/AKT pathway is also responsible for the proliferation of these cells (Gato-Canas et al. 2015). This suggests that AKT is involved in both immune-stimulating and immunosuppressive functions. Multiple recent studies have shown that pharmacologic inhibition of AKT may promote memory T cell formation with activity against cancer cells (Crompton et al. 2015; Waart et al. 2014; Klebanoff et al. 2017). Although AKT is involved in the differentiation of $CD8^+$ T cells, inhibition of AKT has been shown to increase the number of tumor-infiltrating lymphocytes expressing CD8.

$CD8^+$ T cells, or cytotoxic T cells, after an encounter with antigen, are comprised of effector and memory T cells. Almost 90% of effector $CD8^+$ T cells are terminally differentiated following removal of the antigen, with the remaining 10% becoming memory T cells (Klebanoff et al. 2006). This is significant because the quality of the tumor-specific $CD8^+$ T cells is essential to mount an effective antitumor response (Rosenberg et al. 2005). The duration and intensity of AKT activation have a significant impact on the differentiation of the T cell into effector and memory $CD8^+$ T cells (Kim and Suresh 2013). Prolonged constitutive activation of AKT leads to terminal differentiation of effector $CD8^+$ T cells. However, early inhibition of AKT increases the number of memory $CD8^+$ T cells by delaying T cell exhaustion and preserving the naïve $CD8^+$ T cell compartment (Abu-Eid et al. 2014). In adoptive cellular therapy (ACT), these are associated with a greater likelihood of clinical response (Xu et al. 2014; Rosenberg et al. 2011; Louis et al. 2011). ACT is the ex vivo culturing, expansion, and reinfusion of T cells that have been specified with a particular antigen and represents a new strategy in the treatment of advanced cancers (Maus et al. 2014; Jensen and Riddell 2014; Rosenberg and Restifo 2015; Sadelain 2009). Effective T cell transfer is dependent upon the antigen-specific T cells' ability to differentiate into potent effector cells (Falkenburg et al. 2003). AKT activation leads to prolonged T cell receptor signaling, mediated by IL-2 (Macintyre et al. 2011). However, new evidence has demonstrated that AKT inhibition does not impair the growth, survival, proliferation, or glucose metabolism of $CD8^+$ T cells (Finlay 2013). Mitigating excessive AKT-mediating TCR signaling by inhibiting AKT relieves exhaustion and allows the development of antitumor $CD8^+$ T cells with memory phenotype (Chang et al. 2013). These T cells have greater proliferative ability, survival, and cytokine production (Klebanoff et al. 2005). They also

Table 1 Inhibitors of PI3K-Akt pathway in cancer clinical trials

Drugs Involving PI3K-Akt Modulation	Mechanism of Action
Nivolumab	PD-1 inhibition
Pembrolizumab	PD-1 inhibition
Ipilimumab	CTLA-4 inhibition
GSK690693	Competitive ATP inhibition
Uprosertib	Competitive ATP inhibition
Afuresertib	Competitive ATP inhibition
Ipatasertib	Competitive ATP inhibition
Perifosine	Allosteric inhibition
MK2206	Allosteric inhibition
Triciribine	Allosteric inhibition

have higher levels of memory-associated genes such as CD27, CD28, IL-7 receptor, and the lymphoid homing markers CD62L and CCR7 (Cieri et al. 2013; Hinrichs et al. 2011). CD27 is an important costimulatory gene for tumor infiltration, while cells expressing CD62L exhibit an enhanced capacity for survival and proliferation (Louis et al. 2011; Abu Eid et al. 2015; Klebanoff et al. 2011). Cells expressing these markers have superior trafficking capacity to the tumor microenvironment compared to wild-type cells (Klebanoff et al. 2017). In murine models, naïve T cells expanded in the presence of an AKT inhibitor, and the subsequent allogenic transplant delayed tumor growth in melanoma. It also resulted in a lower tumor load and prolonged survival (Waart et al. 2014). In another study of chimeric antigen receptor T cells in the treatment of acute lymphocytic leukemia, CD62L was more highly expressed if grown in the presence of an AKT inhibitor. After implantation, there was a significant improvement in animal survival compared to the control (Klebanoff et al. 2017). The ex vivo inhibition of AKT improves the cell-intrinsic immune and memory qualities of lymphocytes without negatively impacting proliferation and cell survival. Therefore, promoting the expression of antitumor genes through AKT inhibition may have curative potential in ACT (Table 1).

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Conflict of Interest Authors declare that there are no financial or other conflicts of interest exist.

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PI3K Isoform Inhibitors in the Clinic

PI3K Isoform Immunotherapy for Solid Tumours

Jake Scott, Lauren Rees, Awen Gallimore, and Sarah N. Lauder

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Abstract Improving the anti-tumour T cell response as a consequence of immunotherapy can result in eradication of tumour burden, however, the majority of patients fail with current treatment regimens and so novel immunotherapies with greater efficacy and improved tolerability are needed. The phosphoinositide-3-kinase (PI3K) family members that are directly involved in cell signalling comprise PI3Kα, PI3Kβ, PI3Kδ and PI3Kγ, with the latter two isoforms expressed primarily by leukocytes. The survival and optimal function of regulatory T cells (Treg) and

J. Scott · L. Rees · A. Gallimore · S. N. Lauder (✉)
Division of Infection and Immunity, Cardiff University School of Medicine, SIURI, Cardiff C14 4XN, UK
e-mail: LauderSN@cardiff.ac.uk

J. Scott
e-mail: ScottJR1@cardiff.ac.uk

L. Rees
e-mail: ReesLP1@cardiff.ac.uk

A. Gallimore
e-mail: GallimoreAM@cardiff.ac.uk

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myeloid-derived suppressor cells (MDSCs) is dependent on PI3Kδ, whereas tumour-associated macrophages (TAMs), use PI3Kγ. Blocking these signalling isoforms can boost development of effective anti-cancer immune responses and result in control of tumour burden. The dependence on different PI3K isoforms in immune cells makes targeting this pathway an attractive approach for tumour immunotherapy. Herein, we discuss how inhibiting specific PI3K isoforms in pro-tumoural Tregs, MDSCS and TAMs can unleash a powerful anti-tumour immune response, driven by $CD8^+$ T cells, capable of controlling tumour burden and consider how the immune response to therapy needs careful investigation, to identify both the correlates of successful treatment and those that impede the generation of robust anti-tumour responses. Furthermore, we review how combination immunotherapy approaches with both PI3K inhibitors and subsequent immune checkpoint blockade can potentiate the efficacy of monotherapy. Finally, we discuss the recent advances in the use of PI3K isoform-specific inhibitors as an immunotherapy for solid tumours in clinical trials.

1 Introduction

Aberrant PI3K signalling is known to drive cancer progression and common cancers often comprise mutations in *PIK3CA* and loss of PTEN function (reviewed in Thorpe et al. (2015)). A huge effort has been made to develop inhibitors of three broad types; dual PI3K-mTOR inhibitors, pan-class I inhibitors and isoform-specific inhibitors, developed to directly inhibit cancer cell proliferation and survival (reviewed in Janku et al. (2018)), with several currently undergoing testing in clinical studies either alone or in combination with other therapies (Fig. 1). Whilst there is extensive data indicating cancer cell-intrinsic effects of inhibiting PI3K signalling, it is becoming increasingly clear that indirect effects of inhibiting the pathway also contribute to control of tumour growth.

Immunotherapy as a cancer-targeting approach dates back to 1891 when William B. Coley first sought to harness the power of the immune system to control solid tumours (Kienle 2012). The administration of Coley's toxins, a bacterial vaccine, designed to induce a large infection at the tumour site, resulted in clearance of both the infection and regression of the tumour in a proportion of patients. Despite these striking results, the lack of understanding at the time of how the immune system may control cancer, led to these first immunotherapy approaches falling from favour, as surgical, chemotherapy and radiotherapy treatments began to significantly improve cancer outcomes.

The field of immunotherapy has been revived in recent years, with the advent of new treatment strategies such as immune checkpoint blockade (ICB), adoptive transfer T cell therapy (ACT) and tumour vaccine therapy (Reviewed in Waldman et al. 2020). These therapeutic modalities are designed to reinvigorate the anti-tumour immune response, by either directly targeting the T cells themselves, to improve their anti-tumour response, or indirectly, by targeting immuno-suppressive populations, such as regulatory T cells (Tregs), tumour-associated macrophages (TAMs)

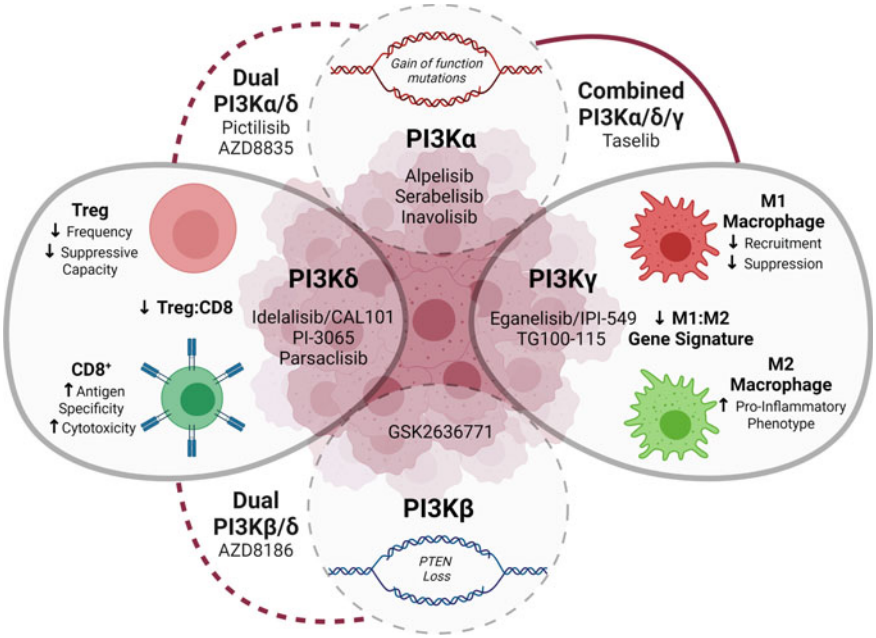

Fig. 1 PI3K isoform-specific inhibitors. Schematic of the PI3K isoform-specific inhibitors currently in various stages of development and their tumour-specific targets

and myeloid-derived suppressor cells (MDSCs), within the tumour microenvironment that typically subdues the T cell response to cancer. Whilst immunotherapy can result in striking control of tumour burden, the number of patients who successfully respond to these therapies remains small. Typically, patients fail to mount a sufficient response to therapy as a consequence of T cells becoming exhausted and dysfunctional prior to or during treatment. As the scientific field continues to elucidate the impediments to T cell anti-tumour responses it is becoming clear that different approaches are still needed, and that a combination of therapies may offer the greatest potential for tumour control.

As discussed in previous chapters, the different PI3K isoforms have critical roles in the signalling of T cells, B cells and innate cells of myeloid origin, such as macrophages. Given the success and tolerability of isoform-specific inhibitors such as the PI3Kδ inhibitor, Idelalisib, for haematological malignancies (Yang et al. 2015), the focus of much research and drug development programmes in recent years has been to understand how other immune cell populations can be targeted via isoform-specific PI3K inhibitors.

Herein, we firstly discuss the data surrounding PI3K isoform-specific inhibition, through genetic inactivation and pharmacological blockade, to target immune cell populations within the tumour microenvironment in preclinical models (Fig. 1). Finally, we discuss the data reported from clinical trials to date, using different inhibitors in solid malignancies.

2 Immune Mechanisms of Action in Preclinical Studies

2.1 PI3Kδ Inhibitors as a Treg Targeted Therapy

FoxP3$^+$ Tregs suppress CD8$^+$ and conventional CD4$^+$ T cells thereby helping to maintain tolerance and prevent autoimmunity. However, these mechanisms in the context of cancer can prevent the host from generating a successful anti-tumour T cell response. Shimizu et al. were the first to demonstrate that removal of regulatory T cells led to the development of potent anti-tumoural CD8$^+$ T cell responses and control of tumour burden (Shimizu et al. 1999). However, complete depletion of regulatory T cells is not considered feasible as a clinical therapy due to the significant presentation of autoimmune side effects and so approaches to selectively inhibit Treg function have been sought. Due to the contrast in reliance on the PI3Kδ isoform for effector T cell and Treg signalling (discussed in Chap. 8 and by Ahmad et al. 2017), PI3Kδ inhibition has been suggested as an attractive approach for Treg-specific cancer immunotherapy.

Initial preclinical studies utilised a strain of mice with either a global or a Treg-specific inactivation of the PI3Kδ isoform (δD910A) to examine the effect that impaired T cell signalling had upon tumour growth. A seminal study by Ali and colleagues demonstrated that δD910A mice were better able to control tumour growth in a number of mouse tumour models (Ali et al. 2014). Adoptive transfer experiments of PI3Kδ-inactive Tregs into wild type tumour-bearing hosts established that the reduced immunosuppression mediated by δD910A Tregs was responsible for reduced tumour growth. The deletion of the CD8$^+$ effector T cell population abrogated tumour control in δD910A mice, demonstrating that tumour control is dependent on both the loss of Treg-mediated immunosuppression and the generation of a robust anti-tumour CD8$^+$ T cell response as a consequence (Ali et al. 2014; Lim et al. 2018). The authors confirmed the therapeutic potential of PI3Kδ, by treating mice with a small molecule inhibitor of PI3Kδ, PI-3065 and demonstrated partial tumour control in both breast and pancreatic cancers *in vivo*. This proof-of-concept study in solid tumours has been strengthened by a number of other groups (Carnevalli et al. 2018; Lauder et al. 2020) who have sought to delineate the mechanism by which PI3Kδ inhibition confers tumour control either alone or in combination with other therapeutic modalities.

Idelalisib, also known as CAL-101 or GS-1101, an approved PI3Kδ inhibitor for haematological cancers (discussed in Chap. 23) and similar PI3Kδ inhibitors are being redeployed by several groups as a therapy designed to specifically target Tregs in solid malignancies. Ahmad and colleagues reported that CAL-101 blockade of PI3Kδ signalling in vitro was critical for Treg suppression and survival, whereas effector CD4$^+$ T cells could utilise PI3Kα and PI3Kβ to maintain their function (Ahmad et al. 2017). Using the Treg-dependent tumour model, TC-1 (lung carcinoma), the authors demonstrated similar findings with modest control of tumour

burden and partial improvement in survival time observed following CAL-101 treatment. However, when they boosted the antigen-specific response using an E7 tumour-specific vaccine in combination with CAL-101, they observed a significant reduction in tumour burden and improved long-term survival compared to either treatment strategy alone. Phenotypic analysis of the anti-tumour immune response in combination-treated animals demonstrated similar findings to the original study by Ali and colleagues (Ali et al. 2014), collectively pointing to a reduction in Treg response and the generation of a robust $CD8^+$ T cell response is necessary for potent tumour control.

Lauder and colleagues expanded on previous studies and demonstrated that whilst all treated mice exhibited a level of tumour control following PI-3065 treatment, there was a dichotomy in the response to therapy, with complete tumour regression occurring in a small proportion of treated animals (Lauder et al. 2020, 2021). Detailed analysis of the anti-tumour immune response generated following PI-3065 treatment supported the previous studies that eradication of tumour burden was reliant on the dampening of the Treg response and the generation of a robust antigen-specific $CD8^+$ T cell response. Whilst all treated mice had a reduced number of tumoural Tregs, those that exhibited only partial control developed a pool of dysfunctional Tregs characterised by increased Ki67, CD69 and LAG3 expression and a reduced number of tumour antigen-specific $CD8^+$ T cells (Fig. 2). Combination treatment with PI-3065 and subsequent anti-LAG3 antibody therapy resulted in significant tumour control in all treated mice (Lauder et al. 2020). However, the greatest significance of the aforementioned study is that it highlighted the tumour-specific impediments to PI3Kδ inhibitors as a therapy. An essential requisite for tumour control was the development of an increased $CD8^+$ T cell: Treg ratio in PI3Kδ responsive tumours. In the absence of an increased ratio, PI3Kδ unresponsive tumours, such as the MC38 colon cancer model, remained unresponsive despite treatment with a secondary immune checkpoint therapy (Lauder et al. 2020, 2021).

In preclinical models that are resistant to Treg-specific inhibition such as MC38, genetic inactivation of PI3Kδ signalling has been reported to enhance tumour growth as a consequence of reduced $CD8^+$ T cell function in vivo (Putz et al. 2012). These conflicting data indicate that simply targeting Tregs may not be sufficient in every tumour type. Indeed, combination immunotherapy approaches designed to target multiple tumour resident populations namely Tregs and TAMs were able to control tumour growth in the previously unresponsive MC38 model (Gyori et al. 2018). Dual PI3K isoform inhibitors offer the potential to target multiple cell types within the tumour microenvironment with a single treatment. Carnevalli et al. compared tumour control in PI3Kδ responsive (CT26 and 4T1) and unresponsive (MC38) tumours to either a PI3Kδ single inhibitor (PI-3065) or a dual PI3Kα/δ inhibitor (AZD8835) (Carnevalli et al. 2018). In all models, AZD8835 offered superior control of tumour growth and prolonged survival. Unlike the previously discussed studies where PI3Kδ isoform inhibitors were routinely administered daily for the duration of the study, the robust anti-tumour response reported with AZD8835 was a consequence of an intermittent dosing regimen, with 2 days on treatment/5 days off treatment. Although not as pronounced as the changes with continual PI-3065 treatment, this intermittent

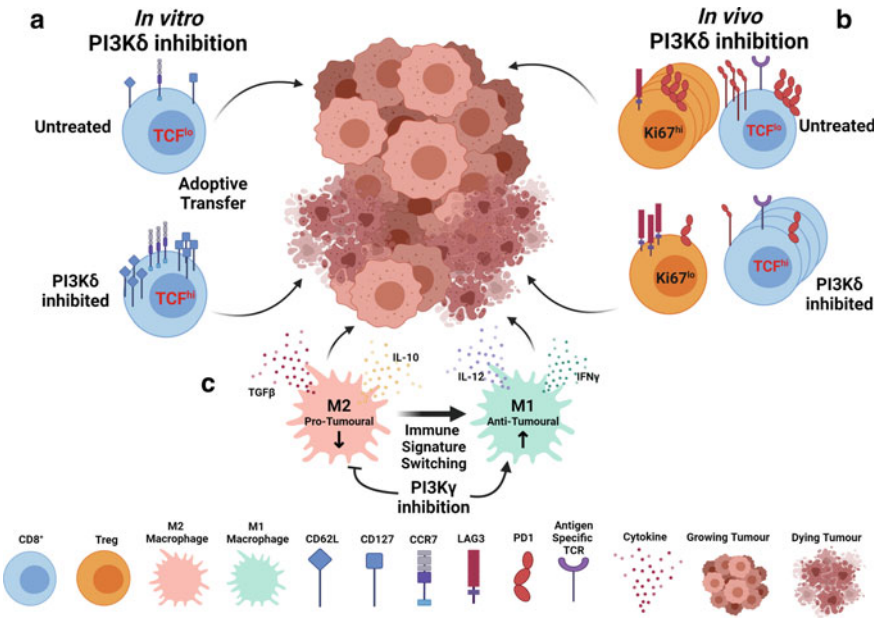

Fig. 2 Immune mechanisms of PI3K specific isoform action. **a** Pharmacological blockade of PI3Kδ in vitro leads to the development of $CD8^+$ T cells with superior anti-tumour activity when adoptively transferred into tumour-bearing hosts. **b** In vivo therapeutic targeting of PI3Kδ results in a reduced Treg: $CD8^+$ T cell ratio and an enrichment in antigen-specific $CD8^+$ T cells with improved anti-tumour function. **c** Specific targeting of PI3Kγ controls tumour growth by inducing immune gene signature switching of macrophages in vivo, from pro-tumoural to anti-tumoural

therapeutic approach resulted in a reduction in Tregs and increased $CD8^+$ T cell: Treg ratio within the tumour. However, AZD8835 conferred enhanced transcriptional and phenotypic changes resulting in $CD8^+$ T cells with increased expression of Ki67, CD25, Granzyme B, IFNγ and a reduced susceptibility to exhaustion as determined by reduced PD1 expression. Furthermore, the intermittent interruption of PI3K signalling in $CD8^+$ T cells appears to promote an IL-2 autocrine signalling loop within the tumour that drives T cell effector function and survival, which ultimately supports tumour control (Carnevalli et al. 2018).

2.2 *PI3Kδ Inhibitors as an Adjuvant to Improve T Cell Therapy in Cancer*

The role of the PI3Kδ isoform in $CD8^+$ T cell signalling during proliferation and effector functions are described in detail in Chap. 12. It is known that pharmacological inhibition of PI3Kδ skews activated $CD8^+$ T cells to develop progeny with a self-renewing phenotype characterised by the expression of the transcription factor TCF1

(Lin et al. 2015; Nish et al. 2017). Several recent studies have demonstrated that the presence of $CD8^+TCF1^+$ stem-like T cells within the tumour microenvironment is associated with tumour control and successful responses to checkpoint therapy (Siddiqui et al. 2019; Kurtulus et al. 2019; Sade-Feldman et al. 2018; Baharom et al. 2021).

Using a preclinical model of melanoma, Bowers and colleagues demonstrated that expansion of $CD8^+$ T cells in the presence of CAL-101 prior to adoptive cell transfer (ACT) into B16F10 tumour-bearing hosts, resulted in significantly reduced tumour burden and prolonged survival (Bowers et al. 2017). RNA-sequencing revealed that CAL-101-treated T cells had improved anti-tumour capacity driven by enhanced expression of TCF1 and a stem-like memory phenotype characterised by increased expression of CD62L, CD127 and CCR7 (Fig. 2). These findings have the capacity to significantly improve cellular immunotherapy approaches such as ACT or chimeric antigen receptor (CAR) T cell therapy, which despite their potential to reinvigorate the anti-tumour immune response, typically fail as a consequence of the transferred cells becoming exhausted and dysfunctional. The generation of human CAR-T cells in the presence of the PI3Kδ inhibitors TGR-1202 or CAL-101 resulted in cells with a less differentiated phenotype compared to untreated cells but with increased cytotoxic capacity in vitro (Dwyer et al. 2020). Such encouraging findings in the preclinical setting warrant the development of clinical trials in patients to determine if PI3Kδ blockade during the manufacture of CAR-T cells can result in a superior tumour control following transfer into patients.

PI3Kδ inhibitors have been also shown to reinvigorate the existing T cell population independently of ACT. Therapeutic administration of the PI3Kδ inhibitor, PI-3065, to mice bearing 4T1 breast tumours promoted the development of a population of stem-like memory T cells, identified by their expression of TCF1, that had superior anti-tumour capacity (Lauder et al. 2020).

2.3 Inhibition of Myeloid Populations Within the Tumour Microenvironment via Specific PI3K Isoform Inhibitors

As discussed in Chap. 6, cells of myeloid origins, such as macrophages, monocytes and neutrophils are reliant on the PI3Kγ isoform for signalling and downstream functions. Within solid tumours, TAMs and MDSCs are typically considered to elicit a pro-tumoural role as they mediate immunosuppression, promote angiogenesis and aid tumour invasion and metastasis (reviewed in Cassetta and Pollard 2018; Groth et al. 2019). High frequencies of TAMs and/or MDSCs within the tumour microenvironment are routinely associated with poor clinical prognosis in a number of cancers (Zhang et al. 2012). Using PI3Kγ specific blockade to target these populations and improve tumour outcomes has been widely studied in recent years with favourable outcomes at the preclinical stage.

Schmid and colleagues were the first to demonstrate that PI3Kγ promotes myeloid cell recruitment to a range of murine tumours driven by the expression of the integrin α4β1 (Schmid et al. 2011). This macrophage recruitment to tumours could be blocked through genetic inactivation (PI3Kγ$^{-/-}$) or pharmacological inactivation with the PI3Kγ specific inhibitor, TG100-115. Consequently, reducing the infiltration of macrophages into the tumour significantly reduced tumour burden. Subsequent studies have sought to delineate further how PI3Kγ inhibition of TAMs contributes to tumour control. Using a mouse model of pancreatic ductal adenocarcinoma (PDAC), TG100-115 was employed to successfully control both primary tumour burden and metastasis (Kaneda et al. 2016a). Kaneda and colleagues demonstrated that inhibition of PI3Kγ altered the immune signature of TAMs, shifting them from an immune-suppressive phenotype by reducing arginase-1, TGFβ, IL-10 and PDGF-BB expression, to a pro-inflammatory phenotype by increasing IFNγ and IL-12. These phenotypic changes to the TAMs resulted in an elevated CD8$^+$ T cell infiltrate into PDAC tumours that promoted tumour control. A parallel study by the same group demonstrated that inhibition of macrophage PI3Kγ promoted NFκB activation whilst impeding C/EBPβ activation. This alternation in transcriptional programme resulted in a switch away from the normal immune suppression driven by TAMS to an anti-tumour immune-stimulatory phenotype (Kaneda et al. 2016b). Whilst PI3Kγ inhibition does not directly target T cells, the switch to a pro-inflammatory phenotype in PI3Kγ-inhibited macrophages indirectly augments the development of a robust anti-tumour T cell response, characterised by increased CD8$^+$ T cell cytotoxicity and Th1 responses driven by increased granzyme B and IFNγ expression and reduced IL-10 (Fig. 2). This enhanced anti-tumour T cell response increased sensitivity to immune checkpoint blockade, with combination therapy (TG100-115 and anti-PD1 antibodies) inducing significantly greater tumour regression ($\geq$80% of treated mice) and long-term survival in comparison to either therapy alone. This sensitivity to immune checkpoint blockade was recapitulated in a study by De Henau and colleagues who demonstrated that high tumoural infiltration of MDSCs was associated with resistance to immunotherapies such as anti-PD1 and anti-CTLA4 antibodies in a number of preclinical models (Henau et al. 2016). Targeting the MDSCs using the specific PI3Kγ inhibitor, IPI-549, restored sensitivity to checkpoint immunotherapy and improved tumour control. Gene expression analysis revealed that IPI-549 treatment resulted in MDSCs with a reduced immune-suppressive phenotype that promoted CD8$^+$ T cell infiltration and an increased CD8$^+$ T cell: Treg ratio within the tumour.

As the studies by Kaneda and colleagues have indicated, not all macrophages within the TME are pro-tumoural. Macrophages with a pro-inflammatory phenotype are considered to be anti-tumoural and contentiously referred to as M1 macrophages whilst M2 macrophages elicit anti-inflammatory effects that promote tumour growth (reviewed in Mantovani et al. 2021). With the previously discussed studies demonstrating that PI3Kγ blockade could switch the transcriptional profile of TAMs from pro-tumoural to anti-tumoural, Lee and colleagues expanded these findings in colon cancer whereby TG100-115 treatment of mice bearing CT26 tumours resulted in a significant reduction in tumour growth (Lee et al. 2020). Analysis of the tumour

microenvironment demonstrated an increased infiltration of M1 macrophages and a reduction in M2 macrophages. Retrospective analysis of a cohort of colorectal cancer patients found that patients with an increased ratio of M1 to M2 macrophages had significantly improved progression-free and overall survival (Lee et al. 2020).

Taken together these studies support the use of PI3Kγ-specific inhibitors as an immunotherapy to reduce both the total number of tumoural TAMS, but also to skew the resident TAM population in favour of M1 macrophages. As PI3Kγ inhibitors reach clinical trials, elucidating the immune contexture of different tumours will highlight the potential for combination therapy with other immunotherapies such as checkpoint inhibitors that could significantly potentiate the effect of either treatment alone.

Despite their preference for PI3Kγ signalling, TAMs and MDSCs can also be therapeutically targeted by blocking the PI3Kδ isoform. In vitro studies demonstrated that CSF-1-induced migration and degradation of the extracellular matrix by TAMs was reduced in the presence of the PI3Kδ inhibitor GS-1101 (Mouchemore et al. 2013). Furthermore, Ali et al. demonstrated that the MDSC expansion driven by the breast tumour cell line, 4T1, is significantly abrogated in D910A mice and the ex vivo capacity of D910A MDSCs to suppress $CD8^+$ T cell proliferation is also potently reduced (Ali et al. 2014). We have found that the therapeutic administration of PI-3065 to 4T1 tumour mice, results in a significant reduction in peripheral expansion of MDSCs (unpublished findings, manuscript in preparation). Furthermore, genetic inactivation of PI3Kδ or oral administration of the PI3Kδ inhibitor IC87114 resulted in reduced recruitment of TAMs to the breast tumour microenvironment, conferring partial tumour control (Goulielmaki et al. 2018). A note of caution when considering these findings is that tumours can drive expansion of MDSCs, therefore, reduced MDSCs in treated animals may be an indirect effect of PI3Kδ inhibition resulting in better control of tumour growth rather than inhibition of PI3Kδ in MDSCs. In addition, Tregs can also drive MDSC expansion hence a reduction in MDSCs may reflect inhibition of Tregs rather than direct effects of the PI3Kδ inhibitor on MDSCs.

Oncolytic viral therapy is a novel cancer immunotherapy approach, whereby viruses are genetically manipulated to specifically target and kill the tumour (reviewed in Harrington et al. 2019). However, despite their high specificity when administered intratumorally, their clinical promise falls short due to low levels of virus reaching the tumour when delivered intravenously (reviewed in Cook and Chauhan 2020). A ground-breaking study by Ferguson and colleagues demonstrated that in vitro treatment of macrophages with the PI3Kδ inhibitor, IC87114 prevented oncolytic viral attachment to the macrophages (Ferguson et al. 2020). Pre-treatment of tumour-bearing mice with IC87114 3 hours prior to administration of the tumour-specific oncolytic virus, resulted in significantly reduced tumour burden, prolonged survival and the development of enhanced anti-tumour immunity as characterised by increased $CD4^+$ and $CD8^+$ T cell tumoural infiltration and elevated numbers of $IFN\gamma^+$ $CD8^+$ T cells.

3 Clinical Trials and Human Studies

Following the success of the first-in-class PI3Kδ isoform-specific inhibitor Idelalisib as a therapy for B cell-derived haematological malignancies, and the preclinical data showing efficacy in solid tumours, multiple clinical trials are now underway with a range of PI3K specific inhibitors in both haematological and solid cancers. Many of the trials for solid tumours use pan-PI3K or isoform-specific inhibitors to treat cancers with activating mutations in the PI3K pathway or loss of PTEN. Details of all trials can be found using the clinical databases: clinicaltrials.gov or eudract.ema.europa.eu; the key trials for isoform-specific inhibitors are also listed in Table 1. However, several trials are now utilising either PI3K inhibitors as an immunotherapy, where the target is the immune cells within the tumour rather than the cancer itself, or as an adjuvant to improve existing immunotherapy approaches, and these trials are discussed in detail below.

3.1 PI3Kδ Inhibitor: Idelalisib

Given the clear role of PI3Kδ in Treg-mediated suppression within the tumour, the potential for repurposing Idelalisib as a therapy for solid tumours has been an attractive prospect. A study sponsored by Gilead (NCT02468557) recently reported its findings from a phase 1 study in pancreatic ductal adenocarcinoma (PDAC). The study intended to primarily assess the safety and adverse event incidence of Idelalisib alone and in combination with other chemotherapy drugs. The study also sought to determine the efficacy of treatment as determined by overall response rate, progression-free and overall survival and immune phenotyping of the tumour environment, specifically the effect Idelalisib had on $CD8^+$ T cells and $FoxP3^+$ Tregs within the tumour. However, the study was terminated early due to two progression-associated deaths and three serious adverse events in the 12 participants enrolled and treated in the Idelalisib only arm (Borazanci et al. 2020). All 12 participants reported adverse events. The toxicity reported is unlikely to be specific to PDAC patients, as serious off-target effects have been widely reported in patients treated with Idelalisib in haematological cancers (reviewed in Cuneo et al. 2019; Hanlon 2020).

A second trial aims to reinvigorate the T cell response in patients who have failed on immunotherapy by using Idelalisib to target immunosuppression within the tumour. Patients with non-small cell lung cancer who have become refractory to anti-PD1 immunotherapy will be treated with a combination of Idelalisib and the anti-PD1 monoclonal antibody, pembrolizumab, to determine if response rates can be improved by dual therapy. As a phase 2 study (*NCT03257722*), the safe and tolerable dose of Idelalisib in combination with a standard dose of pembrolizumab that results in optimal Treg suppression will be established and the efficacy of dual therapy will be measured by the overall response rate to treatment. This study is still in the early stages of recruiting patients and is yet to report any data or safety concerns.

Table 1 PI3K isoform-specific inhibitor clinical trials in solid tumours

PI3K isoform	Drug name	Tumour	Trials		References
PI3Kα	Alpelisib/BYL719	Advanced Solid Tumours Pancreatic Cancer Gastric Cancer Colorectal Cancer Lung Cancer Head & Neck Cancer Ovarian Cancer Oesophageal Cancer	NCT02155088 NCT01613950 NCT01219699 NCT01602315 NCT01719380 NCT01822613 NCT03601507 NCT04729387 NCT02276027 NCT02925234 NCT04753203 NCT04526470	Mono and combination therapy trials	Soares et al. (2018), Juric et al. (2018), Razak et al. (2014), Tabernero et al. (2016), Zhou et al. (2018), Henegouwen et al. (2019)
		Breast Cancer	Licensed for therapy with Fulvestrant		André et al. (2019), Narayan et al. (2021), Juric et al. (2019)
	Serabelisib TAK-117/MLN1117	Advanced Solid Tumours Renal Cancer Endometrial Cancer	NCT02724020 NCT01449370 NCT02725268 NCT03154294	Mono and combination therapy trials	Choueiri et al. (2017), Juric et al. (2017), Scambia et al. (2020), Williams et al. (2020)
	Inavolisib/GDC077	Advanced Solid Tumours	NCT04589845		
PI3Kβ	GSK2636771	Advanced Solid Tumours Melanoma Prostate Cancer Gastric Cancer	NCT04439188 NCT04439149 NCT03131908 NCT02215096 NCT01458067 NCT02615730 NCT02465060	Mono and combination therapy trials	Tawbi et al. (2020), Arkenau et al. (2014)

(continued)

Table 1 (continued)

PI3K isoform	Drug name	Tumour	Trials		References
PI3Kα/δ	Pictilisib/GDC-0941	Advanced Solid Tumours Breast Cance Lung Cancer	NCT00876109 NCT01740336 NCT00876122 NCT00975182 NCT01437566 NCT00960960 NCT00928330 NCT01493843 NCT00974584 NCT02389842	Mono and combination therapy trials	Vuylsteke et al. (2016), Sarker et al. (2015), Krop et al. (2016), Schöffski et al. (2018), Leong et al. (2017)
	AZD8835	Advanced Solid Tumours	NCT02260661	Mono and combination therapy trial	
PI3Kβ/δ	AZD8186	Advanced Solid Tumours Gastric Cancer Prostate Cancer Lung Cancer Breast Cancer	NCT03218826 NCT04001569 NCT01884285	Mono and combination therapy trials	Bono et al. (2018), Hansen et al. (2017)
PI3Kα/δ/γ	Taselib/GDC0032	Advanced Solid Tumours Breast Cancer Lung Cancer	NCT02285179 NCT02785913 NCT01296555 NCT04439175 NCT01862081 NCT02273973 NCT02390427 NCT02457910 NCT02389842 NCT02340221 NCT02154490 NCT02465060	Mono and combination therapy trials	Oliveira et al. (2016), Langer et al. (2019), Abramson et al. (2019), Saura et al. (2019), Filho et al. (2017), Lehmann et al. (2020), Lopez et al. (2019), Dent et al. (2021)
Pan-PI3K	Copanlisib	Advanced Solid Tumours Colon Cancer	NCT03711058 NCT04317105 NCT03502733 NCT03842228	Immunotherapy Combination Trials anti-PD1 (Nivolumab, Durvalumab) anti-CTLA4 (Ipilimumab)	Jakubowski et al. (2020)

Given its unquestionable clinical success for CLL and NHL, many drug companies have invested heavily in developing next-generation PI3Kδ inhibitors that replicate Idelalisib's efficacy but with reduced toxicity. Many of these inhibitors are now being trialled with success for haematological cancers; those that are now being tested in solid tumours are discussed in detail below.

3.2 PI3Kδ Inhibitor: Parsaclisib

Parsaclisib (INCB050465) is an Incyte-developed structurally unique, next-generation PI3Kδ inhibitor, that offers significantly less side effects than first-generation PI3Kδ inhibitors such as Idelalisib (Yue et al. 2019). As a therapy, parsaclisib has shown efficacy in the preclinical Pfeiffer DLBCL model of B cell lymphoma (Shin et al. 2015). A number of clinical trials have reported both efficacy and improved tolerability in haematological cancers (Forero-Torres et al. 2019; Coleman et al. 2021) so its therapeutic potential in solid cancers is now being trialled in patients.

The Incyte sponsored phase 1 trial (NCT02646748), is a two-stage combination therapy trial. In the first stage, escalating doses of parsaclisib were given alongside pembrolizumab to evaluate the safety and tolerability in patients with a range of solid cancers (colorectal, endometrial, breast, pancreatic, lung, head and neck cancer, melanoma). In stage two, efficacy will be assessed in patients with either small cell or non-small cell lung cancer and urothelial cancer. Secondary outcomes will examine how treatment alters the immune contexture of the tumour, specifically examining how the intra-tumoural $CD8^+$ T cell: Treg ratio is altered. The study is due for completion in December 2021, however, preliminary findings indicate that combined therapy significantly reduces the number of intra-tumoural Tregs and increases the $CD8^+$ T cell: Treg ratio (Kirkwood et al. 2018). Furthermore, analysis of both tumour and PBMCs showed increased T cell activation in patients administered combination therapy.

A parallel trial (NCT03589651), also seeks to determine the efficacy of parsaclisib as combination therapy, with the Incyte-developed anti-PD1 monoclonal antibody therapy, retifanlimab, which has shown promise in other clinical trials for solid tumours (Berton-Rigaud et al. 2020). A third trial by the same sponsors (NCT02559492), was designed to determine both the tolerability and efficacy as measured by tumour response rate, progression-free survival and duration of response, to a combination therapy of parsaclisib and the JAK1 inhibitor, itacitinib, in patients with metastatic cancer. After the primary outcome of measuring safety and tolerability of combined treatment groups was completed the study was terminated early, although no data has been published to date.

3.3 Novel PI3Kδ Inhibitors

A Cancer Research UK sponsored phase 2 trial (NCT02540928), sought to examine changes to the $CD8^+$ T cell infiltrate of head and neck squamous cell carcinoma before and after treatment with the PI3Kδ inhibitor, AMG319. An initial report published in 2018, demonstrated between 50 and 88% inhibition of pAKT following treatment with AMG319 (Ottensmeier et al. 2018). However, of the 22 participants recruited, 10 patients reported skin and gut-associated adverse events, with nine participants terminating treatment early. These adverse events are similar to those seen in an earlier clinical trial in CLL and NHL patients treated with AMG319 (Lanasa et al. 2013). As a consequence of the adverse events reported, this study has subsequently been terminated early due to safety concerns, with full results yet to be reported.

The Shanghai Yingli Pharmaceutical developed inhibitor, YY-20394 or Linperlisib, is currently being studied in a number of clinical trials for lymphoma and leukaemia (NCT04108325, NCT04379167, NCT04370405, NCT04279405, NCT04705090, NCT04500561). Interim results from these studies suggest YY-20394 may offer an improved safety profile with less adverse events reported to date (Qiu et al. 2019). YY-20394 is reported to be structurally unique to other PI3Kδ inhibitors, such as Idelalisib. Patients with advanced cancers are currently being recruited to a phase 1 trial (NCT04049929), designed to assess primarily the safety profile of YY-20394 and secondly the efficacy as determined by tumour progression rate.

Finally, the iOnctura developed inhibitor, IOA-244, has reportedly shown great therapeutic potential as a Treg and MDSC targeting therapy in a preclinical model of colon cancer with high Treg: $CD8^+$ T cell ratio (Johnson et al. 2019). IOA-244 combination therapy with either anti-PD1 or anti-PD-L1 significantly inhibited tumour growth. IOA-244 is now being studied in a phase 1 trial (NCT04328844) as a monotherapy and in combination with the chemotherapeutics pemetrexed/cisplatin in a range of advanced solid tumour indications. This first-in-human study will involve a dose escalation to determine the safety profile of IOA-244. The second stage of the study will determine both tolerability and efficacy of IOA-244 as either a mono or combined therapy and will examine changes to the immune phenotype of lymphocytes in peripheral blood. Results from this study are not expected until the middle of 2023 at the earliest.

3.4 PI3Kγ Inhibitor: IPI-549

Given the success of targeting macrophages via PI3Kγ and improving tumour control in the preclinical models discussed earlier, a number of drug companies have advanced PI3Kγ inhibitors into human trials. Infinity pharmaceuticals were the first to test IPI-549, eganelisib, in a cohort of over 200 patients with a range of solid

tumours. The first part of the MARIO-1 (Macrophage Reprogramming in Immuno-Oncology) phase 1 trial (NCT02637531) sought to test the safety and tolerability of eganelisib, in a dose-escalation study, with the efficacy of eganelisib as either a monotherapy or in combination with nivolumab measured in stage two. Full data sets from the study which was due to finish mid-2021 are yet to be released, however, preliminary findings were reported in 2017 and 2018. These preliminary data suggest that eganelisib and nivolumab combination therapy was generally well tolerated with patients typically only experiencing grade 1–2 adverse events (Sullivan et al. 2018; Tolcher et al. 2017). Blood samples taken during the treatment phase indicated T cell activation and reduced immune suppression in peripheral blood. The study sponsors have subsequently commenced two additional trials with eganelisib in 2019. The MARIO-3 phase 2 trial (NCT03961698) is a multi-arm trial in patients with triple-negative breast cancer (TNBC) or renal cell cancer, designed to test the efficacy of targeting macrophages with eganelisib. Patients will receive eganelisib, in combination with either an anti-PD1 (Atezolizumab) or anti-VEGF therapy (Bevacizumab) and the primary outcome of complete response to therapy will be measured over an 18-month period. The secondary outcomes will determine the safety profile, progression-free survival and duration of response. The third Infinity pharmaceuticals sponsored trial is the MARIO-275, phase 2 trial (NCT03980041). Similarly to the MARIO-1 and -3 studies, the efficacy of eganelisib as a monotherapy or in combination with nivolumab will be tested in immunotherapy-naïve advanced urothelial cancer patients.

The efficacy of IPI-549 in a checkpoint inhibitor-independent setting will be tested in patients with TNBC or ovarian cancer (NCT03719326). This two-part dose escalation or expansion study will measure the safety and tolerability of IPI-549, in combination with the dual adenosine receptor antagonist, etrumadenant and the chemotherapy, doxorubicin. Secondary outcomes intend to determine the efficacy of therapy with respect to progression-free and overall survival, duration of response and immune phenotyping of peripheral blood during the study to determine the effect of treatment on the immune response.

A further study, independent of Infinity pharmaceuticals, is designed as a 'poof-of-concept' study to test the hypothesis that macrophage phenotype switching occurs in humans in response to the PI3Kγ inhibitor, IPI-549, as previously reported in preclinical models (Kaneda et al. 2016a, 2016b; Henau et al. 2016). This phase 2 window trial (NCT03795610) in a small cohort of patients with head and neck cancer will take tumour biopsies before and after IPI-549 treatment to allow comparison of the immune signature of TAMs. The secondary objectives aim to determine the safety and tolerability of IPI-549 and to examine changes to the myeloid and T cell tumoural infiltrate following treatment.

3.5 Dual PI3Kδ/γ Inhibitor: Duvelisib

IPI-145, Duvelisib, is one of the next-generation PI3K isoform inhibitors, designed to target both the immune cell dominant PI3Kδ and γ isoforms. A number of clinical trials have reported encouraging results for haematological cancers (O'Brien et al. 2018; Flinn et al. 2019, 2018). Given the success shown with individual δ and γ isoform inhibitors in preclinical models described earlier, the ability to target immunosuppressive Tregs, MDSCs and TAMs with a single agent may offer advanced efficacy but without the toxicity of pan-PI3K inhibitors.

Two parallel studies are currently underway to determine the incidence of adverse events and overall efficacy of Duvelisib and anti-PD1 treatment in patients with head and neck cancer (NCT04193293) or unresectable melanoma (NCT04688658). The effect of Duvelisib and Nivolumab treatment on immune cell function and phenotype in both the tumour and the periphery will be established before, during and after treatment in melanoma patients. Furthermore, the development of PD1 resistance mechanisms will be established by examining the gene signature of tumour-infiltrating immune cells. To date neither study has reported any findings, however, a recent study demonstrated that treatment with IPI-145 provided no control of tumour growth in a mouse model of melanoma (Dwyer et al. 2020). In comparison to treatment with the single δ inhibitors CAL-101 or TGR-1202 or the γ inhibitor IPI-549, T cells treated with IPI-145 were functionally impaired, with less cytokine production and reduced persistence in vivo, suggesting that IPI-145 treatment prevented the generation of a sufficient anti-tumour T cell response capable of controlling tumour burden.

4 Future Perspectives

Targeting the PI3K pathway using isoform-specific inhibitors has demonstrated promise at the preclinical stage, either as a novel monotherapy or in combination with other treatments. Similar to the first-generation immune checkpoint inhibitors that target PD1 and CTLA4 (Nivolumab, Pembrolizumab and Ipilimumab), only tumours that have high numbers of immunosuppressive cells can be successfully treated with PI3K isoform inhibitors. As PI3K isoform-specific inhibitors move into the clinical phase of testing, elucidating the effect of PI3Kδ or PI3Kγ inhibition on the immune response within each tumour type will be critical in determining which patient cohorts are likely to be responsive to PI3K specific therapy. A detailed phenotypic analysis of the tumour-infiltrating lymphocytes has the potential to identify which co-inhibitory receptors could be targeted with additional immunotherapies that could potentiate the clinical response to PI3K inhibitors. However, given the immune-related toxicity reported with both checkpoint inhibitors and first-generation PI3K inhibitors such as Idelalisib, it remains to be seen if PI3Kδ- and PI3Kγ-specific inhibitors will be tolerated sufficiently to enable their future use as a first-line treatment regimen.

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PI3K Targeting in Non-solid Cancer

Hye Na Kim, Heather Ogana, Vanessa Sanchez, Cydney Nichols, and Yong-Mi Kim

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Abstract Despite the therapeutic progress, relapse remains a major problem in the treatment of acute lymphoblastic leukemia (ALL). Most leukemia cells that survive chemotherapy are found in the bone marrow (BM), thus resistance to chemotherapy and other treatments may be partially attributed to pro-survival signaling to leukemic cells mediated by leukemia cell-microenvironment interactions. Adhesion of leukemia cells to BM stromal cells may lead to cell adhesion-mediated drug resistance (CAM-DR) mediating intracellular signaling changes that support survival of leukemia cells. In ALL and chronic lymphocytic leukemia (CLL), adhesion-mediated activation of the PI3K/AKT signaling pathway has been shown to be critical in CAM-DR. PI3K targeting inhibitors have been approved for CLL and have been evaluated preclinically in ALL. However, PI3K inhibition has yet to be approved for clinical use in ALL. Here, we review the role of PI3K signaling for normal hematopoietic and leukemia cells and summarize preclinical inhibitors of PI3K in ALL.

H. N. Kim · H. Ogana · V. Sanchez · C. Nichols · Y.-M. Kim (✉)
Department of Pediatrics, Division of Hematology and Oncology, Children's Hospital Los Angeles, University of Southern California, Los Angeles, California, USA
e-mail: ymkim@chla.usc.edu

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Abbreviations

ALL	Acute lymphoblastic leukemia
BM	Bone marrow
CAM-DR	Cell adhesion mediated drug resistance
CLL	Chronic lymphocytic leukemia
PI3K	Phosphoinotiside-3 kinase
RTK	Receptor tyrosine kinases
GPCRS	G-protein coupled receptors
PIP2	Phosphatidylinositol-diphosphate
PIP3	Phosphatidylinositol-triphosphate
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
PDK1	Phosphoinositide-dependent kinase 1
MSC	Mesenchymal stem cells
T-ALL	T cell acute lymphoblastic leukemia
CNS	Central nervous system

1 Cell Adhesion-Mediated Drug Resistance Triggers PI3K/AKT Signaling in Leukemia

Leukemia originates in the bone marrow and causes uncontrolled expansion of abnormal white blood cells. Leukemia can be categorized into subtypes based on their lineage and the developmental stage affected. Major types of leukemia include acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphoblastic leukemia (CLL), and chronic myelogenous leukemia (CML). Acute leukemia arises when the bone marrow produces immature white blood cells that cannot function properly, and the disease progresses quickly. On the other hand, chronic leukemia involves more mature cells and progresses slower compared to acute leukemia. Lymphoblastic (or lymphocytic) leukemia affects T, B or NK cells whereas myelogenous leukemia derives from granulocytes and monocyte forming cells. Despite much progress over the last decades, the overall survival in patients with acute lymphoblastic leukemia (ALL) is about 40% for adults and 80% for children (Adam et al. 2017; Sanchez et al. 2019; Nikolaenko et al. 2021; Morishita et al. 2012). Although cure rates in children are high, relapse in these patients leads to death in 50–95% of cases (Sanchez et al. 2019) indicating the need for a better understanding of the underlying mechanism for relapse of leukemia cells. Leukemia cells can evade the toxicity of cytoreductive chemotherapy, and the residual ALL cells give rise to relapse. The bone marrow (BM) is the most frequent site of relapse in ALL (Kim et al. 2020; Tabe et al. 2007), and BM relapse is associated with a worse prognosis than isolated extramedullary relapse (Kim et al. 2020; Fuente et al. 2002) indicating that the BM may play a functional role in chemotherapeutic resistance of ALL cells.

Interestingly, BM stromal cell contact has been described to prevent apoptosis of ALL cells (Hoellenriegel et al. 2011; Billottet et al. 2006; Nguyen et al. 2014). In vitro studies show that contact between leukemia and stromal cells and other non-cellular components of the BM promotes cell adhesion-mediated drug resistance (CAM-DR) (Burris 2013; Roversi et al. 2021), which prevents apoptosis of ALL cells (Hoellenriegel et al. 2011; Billottet et al. 2006; Nguyen et al. 2014). Therefore, identifying the underlying mechanism of survival and promotion of CAM-DR may be critical to target chemotherapeutic drug resistance in leukemia (Greenwell et al. 2017a). Drug resistance in B-ALL can be achieved by increased pro-survival intracellular signaling via the PI3K/AKT pathway. The phosphoinotiside-3-kinase (PI3K) pathway is essential for growth, survival, and migration of neoplastic B cells (Greenwell et al. 2017b). It has also been identified as one of the significant pro-survival pathways in CAM-DR: leukemia cell-BM stromal cell contact has been shown to upregulate phosphorylated AKT in B-ALL (Konopleva and Jordan 2011). Despite great interest in inhibition of the AKT pathway in leukemia (Konopleva and Jordan 2011; Shishido et al. 2014; Wang et al. 2004), a clinically available drug for B-ALL treatment remains elusive. Clinically approved PI3K inhibitors have shown long-term side effects (Piddock et al. 2017) indicating challenges remain in designing and implementing PI3K-targeted therapies.

2 The Significance of the PI3K/AKT Pathway in Leukemic Cell

The phosphoinotiside-3-kinase (PI3K) has been implicated in the proliferation and migration of neoplastic B cells (Adam et al. 2017; Sanchez et al. 2019; Nikolaenko et al. 2021). The activation of the PI3K pathway including protein kinase B (AKT) is associated with poor prognosis and drug resistance in pediatric pre-B cell ALL, as well as decreased chemotherapy-driven apoptosis in vitro (Morishita et al. 2012). Stromal cell contact activates the PI3K/AKT signaling pathway in leukemia cells (Kim et al. 2020). PI3K can be activated by receptor tyrosine kinases (RTKs), integrins or G-protein coupled receptors (GPCRS). PI3K can phosphorylate phosphatidylinositol-diphosphate (PIP2) into phosphatidylinositol triphosphate (PIP3), which may activate the serine/threonine kinase AKT. PTEN (phosphatase and tensin homolog deleted from chromosome 10) negatively regulates PI3K signaling (Fig. 1). It has been suggested that integrin-linked kinase (ILK) may be induced in AML cells as a key player upstream of AKT and may be induced upon contact with BM stromal cells (Tabe et al. 2007). Adhesion of integrin alpha 4 to fibronectin was shown to induce activation of the PI3K/AKT pathway and upregulation of anti-apoptotic BCL-2 in leukemia cells (Fuente et al. 2002), resulting in drug resistance. Inhibition of the PI3K/AKT pathway leads to decreased cell proliferation in chronic lymphoblastic leukemia (CLL) (Hoellenriegel et al. 2011) and acute myeloid leukemia (AML) (Billottet et al. 2006; Nguyen et al. 2014), and may sensitize

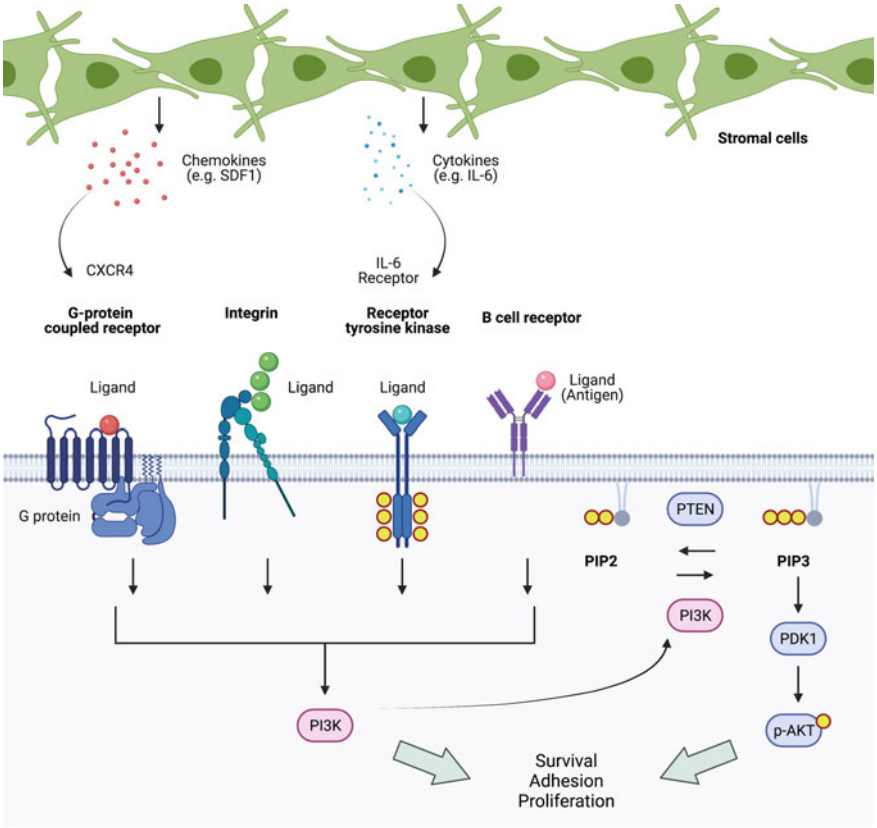

Fig. 1 The phosphoinositide-3-kinases (PI3Ks) signaling pathway. Stromal cell secretion of chemokines and cytokines activates the PI3K/AKT signaling pathway in leukemia cells. PI3K is activated by G-protein coupled receptors (GPCRs), integrins, receptor tyrosine kinases (RTKs), or B cell receptors. PI3K can phosphorylate phosphatidylinositol-diphosphate (PIP2) into phosphatidylinositol triphosphate (PIP3), which may phosphorylate and activate the serine/threonine kinase AKT. PTEN (phosphatase and tensin homolog deleted from chromosome 10) negatively regulates PI3K signaling.

leukemia cells to other treatments (Burris 2013). Disruption of the activation of CXCL12/CXCR4/PI3K/AKT inhibits CXCL12-induced migration of leukemic cell lines and CD34 positive cells from AML patients' bone marrow (Roversi et al. 2021). These findings make the PI3K/AKT pathway an interesting therapeutic target. While the interest in clinical application of PI3K inhibitors is growing, a limitation of PI3K inhibition appears to be side effects that may emerge after treatment (Greenwell et al. 2017a, 2017b).

2.1 Regulation of PI3K/AKT Signaling in Leukemia

Adhesion of ALL cells to BM stromal cells triggers intracellular signals regulating cell adhesion-mediated drug resistance (CAM-DR) (Konopleva and Jordan 2011; Shishido et al. 2014). Stromal cell protection of ALL cells has been shown to require active AKT (Wang et al. 2004). PI3K is activated by cell surface receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs) (Fig. 1). Upon activation, PI3K converts phosphatidylinositol-4,5-bisphosphate (also known as PtdIns(4,5)P2, PI(4,5)P2, or PIP2) into phosphatidylinositol-(3,4,5)-trisphosphate (PtdIns(3,4,5)P3 or PIP3) by phosphorylation. PIP3 recruits PH-domain-containing proteins, such as AKT or phosphoinositide-dependent kinase 1 (PDK1), from cytoplasm toward the intracellular membrane to activate and initiate downstream signaling. AKT is a key signaling molecule in the PI3K pathway. AKT activates downstream signaling molecules, which initiate pro-survival signaling cascades supporting decreased apoptosis and increased survival, cell motility and migration, proliferation, and cell adhesion (Piddock et al. 2017; Park et al. 2010; Luders 1971).

As the PI3K/AKT pathway is integral to survival signaling of leukemia cells, efforts have been made to target this pathway and investigate its effects on leukemia. Tabe et al. showed that blockade of PI3K or ILK signaling with pharmacologic inhibitors LY294002 or QLT0267, respectively, reduced stroma-induced AKT phosphorylation in cells, and ultimately resulted in induction of apoptosis in both leukemic cell lines and in primary AML samples (Tabe et al. 2007). The study highlighted the importance of activation of ILK/AKT, extracellular signal-regulated kinase1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and Notch1/Hes in leukemic NB4 cells cocultured with BM-derived stromal mesenchymal stem cells (MSC). Muranyi et al. demonstrated that targeting ILK and FMS-like tyrosine kinase-3 (FLT3) with an inhibitor of ILK and FLT3, QLT0267, is cytotoxic to AML stem cells using a long-term suspension culture system. Additionally, engraftment of QLT0267 pre-treated patient-derived AML cells was significantly decreased in the BM after 16 weeks of injection compared to DMSO-treated control (Muranyi et al. 2010).

3 Isoforms of PI3K

The PI3K family is categorized into different classes (class I, II, or III) depending on their composition of subunits and functional role in phosphorylating inositol. Only class I PI3Ks can phosphorylate PIP2 and produce PIP3. Class I PI3Ks are further classified into class IA (p110α, p110β, p110δ) and Ib (p110γ). While p110α and p110β subunits are abundantly expressed in tissues, p110δ is expressed in hematopoietic lineage (Piddock et al. 2017; Thorpe et al. 2015; Chantry et al. 1997; Kok et al. 2009). However, differential expression of these subunits can be observed in different cancers. Overexpression of the *PIK3CA* gene encoding the

p110α catalytic subunit is seen in primary AML and multiple myeloma patient samples, and increased detection of p110δ has been shown in breast or melanocytic origin cells (Sawyer et al. 2003). Class II includes three isoforms: PI3KC2α, PI3KC2β, and PI3KC2γ. This class of PI3K synthesizes phosphatidylinositol-3-phosphate (PI(3)P) and phosphatidylinositol-(3,4)-biphosphate (PI(3,4)P2) (Gulluni et al. 2019). PI3KC2α and PI3KC2β are ubiquitously expressed in tissues, while PI3KC2γ is expressed in the liver, prostate, and breast (Wang et al. 2015). Class III PI3K is a heterodimer consisting of a catalytic and a regulatory subunit (Vps34 and Vps15, respectively) (Thorpe et al. 2015) and is involved in protein and vesicle trafficking, autophagy, and mitochondrial lipid catabolism (Thorpe et al. 2015; Backer 2008; Iershov et al. 2019).

3.1 PI3K Mutations in Leukemia

Aberrant activation of PI3K/AKT signaling pathway is often observed in leukemia (Nepstad et al. 2020), thus inhibition of PI3K has been suggested as a potential treatment for leukemic patients (Ecker et al. 2021; Dong et al. 2019; Burger and Wiestner 2018). Constitutive activation of PI3K/AKT via PI3K mutations demonstrates enhanced leukemogenesis. Point mutations in the helical (E542K, E545A) and kinase domain (H1057R) of p110α subunit lead to constitutive activation of PI3K/AKT signaling pathway in the murine hematopoietic cell line Ba/F3. This constitutive activation of PI3K/AKT via p110α subunit point mutations showed leukemogenic potential in vivo (Horn et al. 2008).

Exon 9 and 20 of *PIK3CA* (encoding the p110α catalytic subunit), exon 12 and 13 of *PIKRA* (encoding the p85α regulatory subunit), and exon 2 of *AKT1* mutations have been observed in ALL (Erb et al. 2009). These mutations are more commonly observed in T-ALL relative to B-ALL subtypes (Montano et al. 2018). PI3K/AKT hyperactivation can also be maintained through mutations or deletion of PI3K regulating components such as *PTEN* (Andersson et al. 2015; Bonaccorso et al. 2020; Gutierrez et al. 2009).

4 Preclinical Inhibition of PI3K and PI3Kδ in ALL

There is considerable interest in preclinical and clinical investigation of PI3K inhibitors including pan-PI3K inhibitors targeting all four isoforms of class I PI3K, as well as isoform-selective inhibitors (Fig. 2). Here, we review PI3K inhibitors in preclinical evaluation for the treatment of ALL (Table 2).

Idelalisib

Idelalisib (also known as CAL-101 or GS-1101) inhibits PI3Kδ and has shown to be effective against pre-B acute lymphoblastic leukemia (pre-B-ALL) (Adam et al.

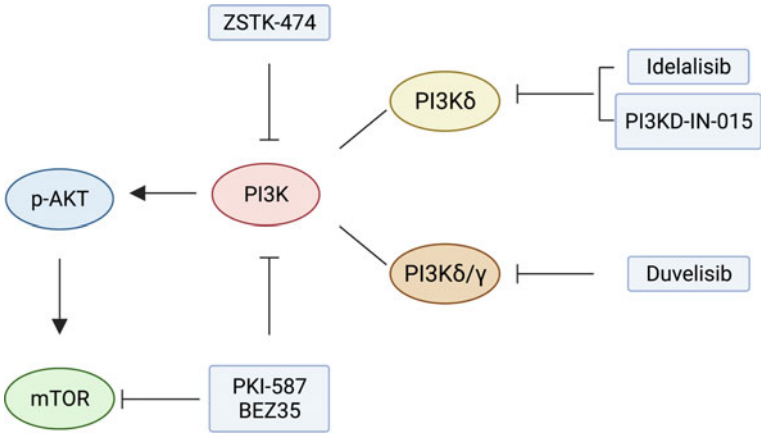

Fig. 2 PI3K pathway inhibitors target one or more aspects of PI3K signaling. PKI-587 and BEZ235 are dual PI3K/mTOR inhibitors. ZSTK-474 is a pan-PI3K inhibitor. Idelalisib and PI3K-IN-015 are each targeting PI3Kδ isoforms. Duvelisib is a dual PI3Kγ/δ inhibitor. Adapted from Sanchez et al. 2019, originally published under CC BY

Table 1 Mutations of PI3K and PI3K regulating components in leukemia

Genetic alterations	Study model	References
E542K, E545A in helical domain H1057R in kinase domain of p110α	Murine hematopoietic cell line	Horn et al. (2008)
Exon 9 and 20 of ***PIK3CA***; Exon 12 and 13 of ***PIKRA***; Exon 2 of ***AKT***	Acute lymphoblastic leukemia	Erb et al. (2009)
PTEN deletion, activating mutations in the *PI3K* and *AKT*	T cell lymphoblastic leukemia	Gutierrez et al. (2009)
PTEN exon 7 mutation	T cell acute lymphoblastic leukemia	Bonaccorso et al. (2020)

2017; Kruth et al. 2017; Tasian et al. 2017; Burger and Okkenhaug 2014; Jou et al. 2002; Fruman and Cantley 2014). PI3Kδ inhibition using idelalisib decreases phosphorylation of AKT levels and in turn prevents migration of B-ALL cells toward SDF-1α in vitro and decreases homing of leukemia to the BM in mice (Adam et al. 2017). Specifically, idelalisib was effective in a subset of B-ALL with TCF3-PBX1 fusion compared to TCF3-PBX1 negative leukemia. This is due to transcriptional regulation of PI3Kδ by TCF3-PBX1, which can bind to the promoter and increase transcription of *PIK3CD* (Eldfors et al. 2017). Idelalisib has also shown anti-leukemic effects in the pre-B-ALL cell line NALM-6, both as a single agent and in combination with doxorubicin (Safaroghli-Azar et al. 2017). Kruth et al. have shown that combination treatment of idelalisib with dexamethasone synergizes and enhances cell death regulatory genes in B-ALL. Combination of idelalisib with conventional cytostatics

Table 2 PI3K inhibitors preclinically evaluated in ALL

Therapeutic drug	Target (s)	Preclinical model	References
Idelalisib (CAL101)	PI3Kδ inhibitor	B-ALL	Adam et al. (2017), Kruth et al. (2017), Tasian et al. (2017), Burger and Okkenhaug (2014), Jou et al. (2002), Fruman and Cantley (2014)
PI3KD-IN-015	PI3Kδ inhibitor	B-ALL	Liu et al. (2016)
Duvelisib (ABBV-954, INK-1197, IPI-145)	PI3Kδ/PI3Kγ inhibitor	B-ALL	Evangelisti et al. (2018)
PKI-587	PI3K/mTOR inhibitor	T-ALL	Gazi et al. (2017)
Dactolisib (BEZ235)	PI3K/mTOR inhibitor	B-ALL	Stefanzl et al. (2017)
ZSTK-474	PI3K pan-inhibitor	T-ALL	Lonetti et al. (2016, 2015)
MK-2206	AKT inhibitor	T-ALL, B-ALL, AML	Richter et al. (2021)

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cytarabine and dexamethasone is also shown to exert anti-proliferative effect in B-ALL (Sklarz et al. 2020). Dual inhibition of PI3K and mTOR in Philadelphia chromosome (Ph)–like B-ALL (Ph-like ALL) decreased leukemia burden in vivo and showed enhanced anti-leukemic effect (Tasian et al. 2017). In an ALL mouse model, inhibition of PI3Kδ decreased integrin alpha 6 expressions on ALL and decreased CNS metastasis (Yao et al. 2018). In T-ALL, shRNA-mediated knockdown of *PIK3CD* or treatment with idelalisib showed reduction in proliferation and increased induction of apoptosis (Yuan et al. 2017).

Copanlisib

Copanlisib is a pan-PI3K inhibitor with preferential binding to p110α and p110β subunits. Copanlisib has shown cell-type-specific cytotoxicity against CLL and diffuse large B-cell lymphoma (DLBCL) (Krause et al. 2018; Lenz et al. 2020). However, in T and B cell lymphoma, copanlisib alone showed dose-dependent cytotoxicity in vitro and synergism with BCL-2 inhibitor venetoclax in xenograft mouse models (Tarantelli et al. 2020).

PI3KD-IN-015

Liu et al. discovered and evaluated PI3KD-IN-015, a selective PI3Kδ inhibitor with moderate effect on proliferation on B cell-related cell lines, CLL, and AML primary cells. Downregulation of PI3K signaling upon PI3Kδ inhibition with PI3KD-IN-015 led to apoptosis and autophagy (Liu et al. 2016).

Duvelisib

Duvelisib, also known as ABBV-954, INK-1197, or IPI-145, is a dual p110γ/p110δ inhibitor that is approved for relapsed and refractory CLL and follicular lymphoma. In BCR-ABL positive B-ALL, duvelisib decreased viability of the cells and induced apoptosis and autophagy in combination with imatinib or nilotinib (Ultimo et al. 2017). In another study, duvelisib, as well as other PI3K inhibitors, sensitized B-ALL cells toward dexamethasone, suggesting p110 inhibition as a promising strategy to improve or overcome glucocorticoid resistance (Evangelisti et al. 2018). Pillinger et al. have shown that PI3Kδ and PI3Kγ inhibition with duvelisib had anti-proliferative activity in primary AML cells. Knockout studies using shRNA showed that p110δ-knockdown caused an anti-proliferative effect on AML cells, whereas p110γ-knockdown revealed decreased AML migration (Pillinger et al. 2016).

CZC24832

Bergamini et al. developed the first PI3Kγ selective inhibitor CZC24832 (Bergamini et al. 2012). Pharmaceutical PI3Kγ inhibition with CZC24832 significantly impaired CLL cell migration, while dual PI3Kδ/γ inhibitor duvelisib had a greater impact than single isoform-selective inhibitors (Ali et al. 2018). This study elucidated the important role of PI3Kγ in malignant B cell migration.

PKI-587

A PI3K/mTOR dual inhibitor PKI-587 (also known as gedatolisib or PF-05212384) shows anti-proliferative effects in T-ALL cell lines in vitro and enhanced survival in immune-deficient mice engrafted with the T-ALL cell line CCRF-CEM in vivo. In mice, PKI-587 delayed tumor progression without inducing adverse side effects, suggesting minimal toxicity profile of the drug (Gazi et al. 2017).

BEZ235

BEZ235, also named dactilosib, is a PI3K/mTOR small molecule inhibitor that led to synergistic anti-proliferative effects on both Philadelphia chromosome (Ph) positive and negative ALL cells when combined with the pan-BCL-2 blocker obatoclax. The use of primary patient samples spanning both Ph+ and Ph− status addresses the important question of the applicability of these drugs to patients with variable cytogenetics (Sanchez et al. 2019; Stefanzl et al. 2017).

ZSTK-474

ZSTK-474, a PI3K pan-inhibitor, decreased T-ALL cell survival and induced apoptosis in nelarabine-resistant T-ALL cells. Investigators showed dephosphorylated AKT and ERK1/2 in response to the combination of nelarabine and ZSTK-474. The dual treatment also demonstrated increased Bax/Bak expression, suggesting efficacy of this drug combination in directly affecting the apoptosis pathway in tumor cells (Lonetti et al. 2016). ZSTK-474 showed the highest cytotoxic effects when compared to selective isoform inhibition (using the p110α inhibitor A-66, p110β inhibitor TGX-221, p110δ inhibitor CAL-101, and p110γ inhibitor AS-605240) or the dual p110γ/δ

inhibition (using duvelisib) in T-ALL cell lines (Lonetti et al. 2015). When examining the mechanism of action of ZSTK-474, investigators observed a decrease in phosphorylation of AKT at both activation residues (Thr 308 and Ser 473) (Sanchez et al. 2019).

MK-2206

Simioni et al. first evaluated the AKT allosteric inhibitor MK-2206 in T-ALL cell lines and primary cells and demonstrated cell cycle arrest, apoptosis and autophagy, and also cytotoxicity in primary T-ALL initiating cells (Simioni et al. 2012). The group found that MK-2206 treatment caused dephosphorylation of AKT and its downstream targets, GSK3α/β and FOXO3A. MK-2206 was shown to inhibit B-ALL cell proliferation and sensitized B-ALL cells to the BCL-2 inhibitor venetoclax (Richter et al. 2021). In AML cell lines, MK-2206 induced cell cycle arrest, suppressed anti-apoptotic myeloid cell leukemia-1 (Mcl-1), and increased GSK3β activation (Lu et al. 2015). The study also showed that MK-2206 enhanced the cytotoxicity of cytarabine and suggested synergistic effects. However, Konopleva et al. reported that when administered to adult AML patients in a phase II clinical trial (NCT01253447), 1 out of the 18 evaluable patients in the study showed complete remission and six of the 18 patients experienced drug-related toxicity presenting as a pruritic rash. There were only modest decreases in the phosphorylation of AKT at Ser 473, and the insufficient anti-leukemia effects led to early study termination (Konopleva et al. 2014).

5 PI3K Inhibitors in Clinical Trial in ALL

As efforts to develop new preclinical and clinical inhibitors of the PI3K pathway are ongoing, few PI3K targeting drugs are under clinical evaluation for the treatment of acute lymphoblastic leukemia (Table 3). Buparlisib (BKM120) is a pan-class I PI3K inhibitor that showed modest efficacy and was tolerable in advanced acute leukemias (12 acute myeloid leukemia, one acute lymphoblastic leukemia, and one mixed phenotype leukemia) (Ragon et al. 2017). Severe adverse events were reported for buparlisib in combination with fulvestrant as tested in breast cancer patients (Leo et al. 2018). Copansilib is scheduled to be evaluated in ALL (NCT04803123). Idelalisib (CAL-101) testing in ALL and AML was terminated because the study was unable to recruit eligible subjects (NCT01620216). BEZ235 (also known as dactolisib) is under clinical evaluation at the Johann Wolfgang Goethe University Hospital in Germany for use in ALL and other hematological malignancies (NCT01756118). MK-2206 was evaluated in phase II clinical trials in adult AML but was terminated due to insufficient effects at tolerable doses (NCT01253447). PI3K inhibitors have yet to progress far enough to reach FDA approval for leukemia.

Table 3 PI3K inhibitors in clinical trial in ALL

Therapeutic drug	Target (s)	Clinical trial status	Disease	Clinical trial number	Outcome
Buparlisib (BKM120)	Pan-PI3K inhibitor	Phase I	AML, ALL	NCT01396499	Completed
Copansilib	Pan-PI3K inhibitor	Phase I	ALL	NCT04803123	Not yet recruiting as of March 2021
CAL-101	PI3Kδ inhibitor		ALL, AML	NCT01620216	Terminated (Unable to recruit enough eligible subjects)
Dactolisib (BEZ235)	PI3K/mTOR inhibitor	Phase I	ALL, AML	NCT01756118	Unknown
MK-2206	AKT inhibitor	Phase II	AML	NCT01253447	Terminated

6 PI3K Targeting Drugs in Clinical Trials

Although there are no FDA-approved PI3K inhibitors for acute leukemias, so far three PI3K inhibitors have gained approval for treatment of other cancers: Idelalisib (inhibitor of PI3Kδ), Copansilib (Pan-class I PI3K inhibitor), and Duvelisib (dual inhibitor of PI3Kδ and PI3Kγ) (Nikolaenko et al. 2021).

Idelalisib (Zydelig) was approved for patients with indolent non-Hodgkin's lymphoma (iNHL) in July 2014 (Miller et al. 2015) after showing a promising result in a single-arm phase II study. In this study, idelalisib showed an overall response rate (ORR) of 54% for patients with follicular lymphoma and 58% for those with SLL (Gilbert 2014; Furman et al. 2014). However, idelalisib has been associated with pulmonary adverse events (Migault et al. 2018; Barr et al. 2016) and other side effects (Rhodes et al. 2018; Mauro et al. 2018).

Copanlisib was FDA-approved in September 2017 for adult relapsed follicular lymphoma patients [88, 89]. It is the second PI3K inhibitor approved and the first intravenous PI3K inhibitor approved. Although it is a pan-class I PI3K inhibitor, it predominantly acts against PI3Kα and PI3Kδ. Copanlisib has higher affinity for the p110 isoforms and decreased gastrointestinal toxicity compared with idelalisib (Krause et al. 2018).

Duvelisib is an oral dual inhibitor of PI3Kδ and PI3Kγ that is approved for relapsed and refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) and follicular lymphoma patients (Flinn et al. 2018a; 2018b; O'Brien et al. 2018; Duvelisib approved for leukemia, Lymphoma. 2018; Blair 2018). The FDA approval was based on favorable outcomes of the phase III DUO and the phase II DYNAMO trials (Flinn et al. 2018b; Flinn et al. 2016).

7 Discussion

The PI3K/AKT pathway is a central hub in drug resistance of leukemia, particularly in CAM-DR, involving several downstream signaling pathways that support survival and proliferation of leukemia cells. It remains an attractive therapeutic target because of this central role. Concerns about short-and long-term toxicities of FDA-approved PI3K inhibitors seem to warrant early recognition and treatment of PI3K targeting related toxicities (Hanlon and Brander 2020). Increasing numbers in preclinical candidate drugs support achieving the ultimate goal of overcoming PI3K/AKT-mediated drug resistance. Further studies to understand the role of the PI3K isoforms and how to target isoforms or pan-PI3K safely are needed to finally develop new therapies in ALL.

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AKT Isoforms as a Target in Cancer and Immunotherapy

Daniel J. Smit and Manfred Jücker

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Abstract Over the past years, targeted therapies have received tremendous attention in cancer therapy. One of the most frequently targeted pathways is the PI3K/AKT/mTOR signaling pathway that regulates crucial cellular processes including proliferation, survival, and migration. In a wide variety of cancer entities, the PI3K/AKT/mTOR signaling pathway was found to be a critical driver of disease progression, indicating a noteworthy target in cancer therapy. This chapter focuses on targeted therapies against AKT, which is a key enzyme within the PI3K/AKT/mTOR pathway. Although the three different isoforms of AKT, namely AKT1, AKT2, and AKT3, have a high homology, the isoforms exhibit different biological functions. Recently, direct inhibitors against all AKT isoforms as well as selective inhibitors against specific AKT isoforms have been extensively investigated in preclinical work

D. J. Smit · M. Jücker (✉)
Institute of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
e-mail: juecker@uke.de

D. J. Smit
e-mail: d.smit@uke.de

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as well as in clinical trials to attenuate proliferation of cancer cells. While no AKT inhibitor has been approved by the FDA for cancer therapy to date, AKT still plays a crucial role in a variety of treatment strategies including immune checkpoint inhibition. In this chapter, we summarize the status of AKT inhibitors either targeting all or specific AKT isoforms. Furthermore, we explain the role of AKT signaling in direct inhibition of tumor cell growth as well as in immune cells and immune checkpoint inhibition.

Abbreviations

AKT	AKT8 virus oncogene cellular homolog
ATP	Adenosine triphosphate
AO	Antisense oligonucleotide
Bcl-xL	B cell lymphoma-extra large
BRAF	V-raf murine sarcoma viral oncogene homolog B1
COSMIC	Catalog of Somatic Mutations in Cancer
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
CRC	Colorectal carcinoma
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
GSK3ß	Glycogen synthase kinase 3 beta
HER2	Human epidermal growth factor receptor 2
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
ITIM	Immunoreceptor tyrosine-based inhibition motif
ITSM	Immunoreceptor tyrosine-based switch motif
JAK	Janus kinase
kDa	Kilodalton
MEK	MAPK/ERK Kinase
MHC	Major histocompatibility complex
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC1	MTOR complex 1
NCT	ClinicalTrials.gov identifier
NSCLC	Non-small-cell lung carcinoma
OS	Overall survival
PD1	Programmed cell death protein 1 receptor
PDK1	Phosphoinositide-dependent kinase-1
PH	Pleckstrin homology domain
PI-3,4,5P_3	Phosphatidylinositol-3,4,5-trisphosphate
PI3K	Phosphoinositide-3-kinase

PP2A	Protein phosphatase 2
PRAS40	Proline-rich AKT substrate of 40 kDa
PTEN	Phosphatase and tensin homolog
Raf	Rat fibrosarcoma
Ras	Rat sarcoma
RTK	Receptor Tyrosine Kinase
S6K1	Ribosomal protein S6 kinase beta 1
SHP2	Src homology region 2 domain-containing phosphatase 2
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TSC2	Tuberous sclerosis complex 2

1 PI3K/AKT/mTOR Signaling in Cancer

In cancer, an overactivated PI3K/AKT/mTOR signaling pathway is commonly identified (Yuan and Cantley 2008). While mutations in *PIK3CA*, the gene encoding for the p110α catalytic subunit of PI3K, occur frequently in endometrial (21.86%), breast (19.01%) and large intestine cancer (12.15%) (COSMIC 2020a), mutation rates of AKT and mTOR are lower (Zhang et al. 2017).

However, mutational iteration in downstream elements can also be observed. In the mammalian genome there are three known isoforms of AKT that are referred to as AKT1 (PKBα), AKT2 (PKBβ), and AKT3 (PKBγ) (Datta et al. 1999). Each enzyme isoform is encoded on a separate gene in different chromosomes. The *AKT1* gene is located on chromosome 14 (14q32), the *AKT2* gene on chromosome 19 (19q13), and the *AKT3* gene on chromosome 1 (1q44) (Cheng et al. 1992; Nakatani et al. 1999; Staal et al. 1988). Although AKT2 and AKT3 isoforms share 81% and 83% amino acid sequence homology with AKT1, respectively (Vanhaesebroeck and Alessi 2000), their functional roles are yet different, and overlapping as well as non-overlapping roles have been described (Grottke et al. 2016; Hinz and Jucker 2019). Comparing tissue distribution, Bellacosa et al. have demonstrated that AKT1 and AKT3 are expressed widely in a variety of human tissues, whereas AKT2 expression is limited to insulin-responsive tissues (Bellacosa et al. 2004). A recent in vivo study introducing a homozygous *Akt* isoform-specific knockdown confirmed the abovementioned results by Bellacosa et al. (Linnerth-Petrik et al. 2016). In *Akt1* $^{-/-}$ mice, impaired growth could be observed, while *Akt2* $^{-/-}$ mice showed an insulin-resistant phenotype, comparable to type 2 diabetes (Garofalo et al. 2003; Linnerth-Petrik et al. 2016; Yang et al. 2003). Homozygous knockdown of *Akt3* leads to a decrease in brain size in animal models (Yang et al. 2005).

Alterations in AKT activity are commonly observed in cancer. Staining for phospho-AKT Ser 473, which is one of the two phosphorylation sites that are required

for full AKT activation could be detected in a wide range of tumors. Moreover, active AKT correlated with poor prognosis (Osaki et al. 2004). On the contrary, a recently published study on triple-negative breast cancer showed that downregulation of *AKT3* increases tumor cell migration and metastasis by upregulating S100A4 in vivo (Grottke et al. 2016). These data indicate that even opposing effects of the same AKT isoform in different tumor entities are possible and signal transduction of PI3K/AKT/mTOR pathway is most likely is not linear and interactions with other pathways widely occur (Manning and Toker 2017).

The catalog of somatic mutations in cancer (COSMIC) lists the highest percentage of mutated *AKT1*, *AKT2,* and *AKT3* in primary patient-derived tumor samples with 8.13% in meningeal cancer (*AKT1,* (COSMIC 2020b)), 3% in liver cancer (*AKT2,* (COSMIC 2020c)) and 11.21% in liver cancer (*AKT3*, (COSMIC 2020d)). Compared to the abovementioned mutation rates of *PIK3CA* (COSMIC 2020a), the frequency of mutations in AKT isoforms is substantially lower. Nevertheless, overexpression of all three AKT isoforms has been found in different cancers including the leading tumor entities such as lung cancer, breast cancer, prostate cancer, and colorectal cancer (Bray et al. 2018; Osaki et al. 2004).

2 AKT as Prognostic Marker in Cancer

Constitutive activation of AKT, i.e., its phosphorylated form, has been described as a negative prognostic marker in pancreatic cancer (Massihnia et al. 2017), NSCLC (Qiu et al. 2013), and breast cancer (Pérez-Tenorio and Stål 2002). However, distinct functional roles with even contradictory effects have been described for AKT isoforms in breast cancer (Hinz and Jucker 2019). Despite the high genetic homology of roughly 80% and the regulation by the same upstream elements, different conclusions could be drawn with respect to the prognostic role of phosphorylated AKT and expression of AKT isoforms.

While high levels of phosphorylated AKT1 in breast cancer cells result in impaired overall survival (OS) and resistance to chemotherapy regimens (Liang et al. 2006; Liu et al. 2012), high expression of AKT2 in HER2 positive (Grell et al. 2012) and tamoxifen-treated ER positive breast cancer (Kirkegaard et al. 2005) is associated with increased OS. In contrast to the beneficial impact of AKT2 in ER positive breast cancer on hormone therapy sensitivity, a high expression of activated AKT3 mediates resistance to tamoxifen treatment (Faridi et al. 2003).

3 Inhibitors of AKT

The vast majority of AKT inhibitors inhibit all three AKT isoforms. AKT inhibitors can be divided into groups depending on their chemical structure and based on their mechanism of action. Most inhibitors act in an ATP-competitive manner, directly

targeting the ATP binding site or active center of AKT. ATP-competitive AKT1/2/3 inhibitors include Triciribine (also referred to as AKT inhibitor V, PTX-200, API-2), GSK2141795 (Uprosertib), GSK690693, LY2780301, AT13148, AT7867, AZD5363 (Capivasertib), GDC0068 (Ipatasertib) and GSK2110183 (Afuresertib). In contrast, MK-2206, ARQ 092 (Miransertib), Perifosine, Miltefosine, and ARQ 751 act as allosteric inhibitors of AKT, therefore binding outside the active center. All of these inhibitors are currently limited to preclinical evidence and clinical trials, while Ipatasertib is the most advanced drug and is in a phase III clinical trial. No inhibitor of AKT has been approved by the FDA for cancer therapy to date (Table 1).

3.1 ATP Competitive AKT1/2/3 Inhibitors

3.1.1 Triciribine

Triciribine was first synthesized in 1971 (Schram and Townsend 1971) and its molecular derivates are nowadays also referred to as AKT inhibitor V, PTX-200, or API-2. Preclinical studies could identify Triciribine derivate API-2 as a highly selective inhibitor of AKT, without cross-reaction with other kinases including PI3K, PDK, STAT3, and ERK1/2 (Yang et al. 2004). The group revealed a strong antitumoral activity in different types of cancer including ovarian, prostate, colorectal, breast cancer, and melanoma cell lines in vitro (Yang et al. 2004). Further analysis in a xenograft mouse model confirmed the results observed in vitro and concluded that API-2 exhibits a potent antitumoral activity, especially in cancers that display AKT signaling activation (Yang et al. 2004). The two available phase II studies for Triciribine in ovarian cancer (PTX 200 and carboplatin, NCT01690468) and locally advanced and metastatic breast cancer (Triciribine-phosphate, Paclitaxel, Doxorubicin Hydrochloride, and Cyclophosphamide, NCT01697293) have been terminated early due to a change in strategic focus and the lack of patients eligible for study inclusion, respectively. Currently there is one further trial in phase II examining PTX-200 (Triciribine) plus Cytarabine in refractory or relapsed acute leukemia (NCT02930109).

3.1.2 GSK2141795

GSK2141795 (also referred to as Uprosertib) is a pan-AKT inhibitor with an ATP-competitive mechanism of action. Although GSK2141795 does not selectively inhibit any particular AKT isoform primarily, the authors report less impact of this novel AKT inhibitor on glucose metabolism compared to other pan-AKT inhibitors, which was related to fewer off-target effects of the AKT inhibitor on AMP-activated protein kinase and protein kinase C isoforms (Dumble et al. 2014). The drug potently inhibited cancer cells with known AKT activating mutations in a xenograft mouse model. However, the drug did not exert its potent antitumoral activity in cells with activated

MAPK pathway. The authors overcome this resistance by dual targeting of AKT and MEK using GSK2141795 in combination with GSK2110212 (Dumble et al. 2014).

The drug entered several clinical trials either alone or mostly in combination with other kinase inhibitors such as trametinib in a wide variety of cancer entities including gynecological tumors and cancers of other origins in UICC stage IIIC-IV. Although the drug promised great sensitivity within the nanomolar range in preclinical testing (Dumble et al. 2014) in combination with MEK inhibitor trametinib inhibiting several cell lines, preferentially those harboring an activating AKT mutation, the drug dashed expectation 2014in a dose-escalating phase I clinical trial. In particular, the drug combination exhibited low tolerability and minimal clinical activity in triple-negative breast cancer and BRAF-wild type advanced melanoma (Tolcher et al. 2020).

3.1.3 LY2780301

LY2780301 is an ATP-competitive inhibitor of AKT1/2/3 and mTORC1 downstream effector ribosomal S6 kinase (S6K1) (also referred to as p70S6K). The inhibitor exhibited strong efficacy in ovarian cancer, colorectal cancer, prostate cancer, and lung cancer xenograft preclinical models (Azaro et al. 2015). The drug entered phase I and phase II clinical trials in 2010 and 2013, respectively. Only one phase II clinical trial (NCT01980277, "TAKTIC") of LY2780301 in combination with chemotherapeutic paclitaxel for HER2 metastatic breast cancer is currently listed on clinicaltrials.gov. Although Vicier et al. stated that the combination was tolerable in locally advanced and metastatic breast cancer patients with potential efficacy (Vicier et al. 2019), the clinical trial was not further conducted and drug development was terminated in 2018. Unfortunately, no justification for drug development termination was reported.

3.1.4 AT13148

AT13148 belongs to the family of ACG kinase inhibitors that inhibits AKT and ROCK kinases (Yap et al. 2012). The drug entered a phase I clinical trial in 2012 and results were published in 2020 by Mcleod et al. (2020). The study groups consisted of patients with solid tumors, with breast and colorectal cancers being the most common subtype. In total 52 participants received at least one dose of AT13148. The study revealed a narrow therapeutic index for the multi-kinase targeting inhibitor that resulted in termination of drug development (McLeod et al. 2020).

3.1.5 AT7867

AT7867 (Grimshaw et al. 2010), similar to LY2780301, targets AKT and S6K1 in an ATP-competitive way. Preclinical work demonstrated growth inhibition of the uterine sarcoma cell line MES-SA and the human glioblastoma cell line U87MG

in vitro as well as in vivo in xenograft models after subcutaneous injection of the aforementioned cells in BALB/c mice (Grimshaw et al. 2010). However, the drug was never further examined in clinical trials.

3.1.6 AZD5363

AZD5363, also referred to as Capivasertib, acts as an ATP-competitive inhibitor of all three AKT isoforms (Davies et al. 2012). The drug achieved a dose-dependent inhibition of tumor growth and additionally enhanced the inhibitory effect of the TKI inhibitor lapatinib, chemotherapeutic Doxetaxel, and the HER2 antagonist trastuzumab in a xenograft mouse model after injection of BT474 breast cancer cells into SCID mice (Davies et al. 2012). Recent findings from the FAKTION trial (NCT01992952) indicate a beneficial effect of Capivasertib in combination with estrogen receptor antagonist fulvestrant on progression-free survival of ER positive breast cancer patients (Jones et al. 2020). The drug combination will be further examined in a phase III trial. Further phase III clinical trials are currently examining Capivasertib in combination with paclitaxel as a first-line treatment regimen for triple-negative breast cancer (NCT03997123) and Capivasertib with fulvestrant in hormone receptor-positive breast cancer (NCT04305496).

3.1.7 GSK2110183

GSK2110183, also referred to as Afuresertib, is an ATP-competitive pan-AKT inhibitor that was first described in 2012 simultaneously with GSK2141795 (Uprosertib) (Dumble et al. 2014). The drug was examined in phase I and phase II trials for the treatment of leukemia, multiple myeloma, and solid tumor entities. Only one phase II clinical trial (NCT01532700) including 28 chronic lymphocytic leukemia patients treated with the monoclonal CD20 antibody Ofatumumab plus GSK2110183 had reported results. Out of 28 patients, 14 participants had stable disease, 13 participants developed a partial response, and one participant a complete response to treatment with both drugs. The other studies were either terminated by the sponsor (NCT02177682), by physician decision (NCT01531894) or had no results reported after the completion of the study.

3.1.8 GSK690693

GSK690693 was first described in 2008 by Rhodes et al. as a potent pan-AKT inhibitor that acts in an ATP-competitive manner (Rhodes et al. 2008). In vitro assays on cancer cell lines conducted by the aforementioned group revealed an IC_{50} ranging from 2–13 nM for the three AKT isoforms, indicating a high potency. Additionally, in vivo xenograft experiments after subcutaneous injection of several breast cancer cell lines (HCC1954, MDA-MB-453, BT474), the prostate cancer cell line LNCaP,

the pancreatic tumor cell line PANC1, and the ovarian cancer cell line SKOV3 in immunocompromised mice confirmed the inhibition of tumor growth after treatment with GSK690693 (Rhodes et al. 2008). The drug was tested in a first in human phase I clinical trial including 70 participants (NCT00493818). However, the study was terminated in 2017 and no results were published (NCT00493818). A further study with GSK690693 in refractory or relapsed hematopoietic malignancies was planned but withdrawn before enrollment (NCT00666081).

3.1.9 GDC-0068

GDC-0068 also referred to as Ipatasertib, developed by Roche, was first published in 2017 reporting the results of a phase Ib study including 52 patients suffering from advanced solid tumors including metastatic breast cancer, colorectal cancer, and prostate cancer among other solid tumor entities (Saura et al. 2017). The drug is currently under investigation in four phase III studies for breast cancer and prostate cancer patients (Clinical Trials Database 2020a). Data from the randomized phase II trial "FAIRLINE", testing neoadjuvant Ipatasertib and Paclitaxel vs. Placebo and Paclitaxel in triple-negative breast cancer, revealed that, although the pathological outcome was not significantly influenced by the addition of Ipatasertib, the intervention group still showed benefit in clinical imaging (Oliveira et al. 2019). The rates of overall response before surgery, measured by magnetic resonance imaging, were significantly higher after treatment with Ipatasertib (Oliveira et al. 2019). In addition, the authors reported a better response rate in patients with *PIK3CA*, *PTEN* or *AKT1* mutated tumors, highlighting the benefits of personalized cancer therapy. Nevertheless, the rate of adverse events, especially of serious adverse events, was higher in the Ipatasertib/Paclitaxel group (13.2% vs 4%) (Oliveira et al. 2019).

3.1.10 ARQ 092 and ARQ 751

ARQ 092 (Miransertib) and its next-generation derivate ARQ 751 are selective ATP-competitive inhibitors of all three AKT isoforms (Landel et al. 2020) that are currently examined in phase I clinical trials for the treatment of solid tumors including endometrial cancer and other incurable solid tumors who failed standard therapy (NCT02476955, NCT01473095). Additional phase I clinical trials are available for patients with solid tumors with mutations in *PIK3CA*, *AKT,* or *PTEN* (NCT02761694) and recurrent malignant lymphoma (NCT01473095).

3.2 Allosteric AKT1/2/3 Inhibitors

In contrast to the abovementioned ATP-competitive inhibitors of AKT, allosteric inhibitors do not interfere with the active binding side nor act in an ATP-competitive

manner. Furthermore, allosteric inhibition of AKT has been suggested not to hyperactivate AKT (Okuzumi et al. 2009) and therefore prevents subsequent upregulation of downstream elements including mTOR. Additionally, allosteric inhibition of AKT results in less adverse events compared to ATP-competitive inhibition (Landel et al. 2020). In the following section, allosteric inhibitors of AKT will be discussed.

3.2.1 MK-2206

MK-2206 acts as an allosteric AKT inhibitor (Hirai et al. 2010). The drug is currently in phase II clinical trials in patients suffering from solid tumor entities including advanced breast cancer and advanced colon and rectum carcinoma (Clinical Trials Database 2020b). First trials started in 2010; nowadays some phase II trials are already completed, with results partly published. In total 31 trials are listed in the clinical trials database of the United States National Library of Medicine. However, no phase III or IV studies are listed. As an allosteric inhibitor of AKT, MK-2206 does not compete with ATP binding nor the substrates of AKT (Lindsley et al. 2008). Allosteric inhibition by MK-2206 stabilizes the inactivated AKT conformation and prevents activation by PDK1 (Lindsley et al. 2008), as evidenced in in vitro experiments as well as in a xenograft mouse model after subcutaneous cell injection of cholangiocarcinoma cells in vivo, which evaluated Thr 308 and Ser 473 phosphorylation of AKT as a readout for AKT activation after application of MK-2206 (Ewald et al. 2013). Lindsley et al. presented a model of inhibition that is based on strong protein–protein interactions mediated by MK-2206 between the PH and catalytic domains and thus a stabilization of the inactive AKT (Lindsley et al. 2008). Lack of phosphorylation at the abovementioned amino acid residues suggests that AKT remains inactive and does not catalyze phosphorylation of downstream targets such as TSC2. In the absence of AKT-mediated phosphorylation, TSC2 is active as a tumor suppressor, inhibiting mTOR and therefore preventing protein synthesis to occur (Martini et al. 2014). Although the inhibitor did not succeed in clinical trials, a vast amount of in vitro and in vivo studies targeting AKT using either MK-2206 alone or in combination with another drug targeting the PI3K/AKT/mTOR signaling pathway have been conducted (Ewald et al. 2013; Schulte et al. 2020). Frequently used targets for dual inhibition are downstream elements of AKT including mTOR using RAD001 (Ewald et al. 2013; Grabinski et al. 2012; Hinz et al. 2021; Ji et al. 2014; Smit et al. 2020) or proteins within related signaling pathways including RAS/RAF/MEK/ERK signaling pathway (Ewald et al. 2015, 2014; Schulte et al. 2020).

3.2.2 ONC201 (TIC10)

TIC10, also referred to as ONC201, indirectly inactivates AKT and MEK through Foxo3a dephosphorylation and *TRAIL* upregulation and demonstrated anti-tumor activity of TIC10 in cancer cell lines, primary patient samples, and xenograft models (Allen et al. 2013). Remarkably, in contrast to other drugs described in this chapter,

the mechanism of action includes the activation of an endogenous inhibition pathway following *TRAIL* upregulation (Allen et al. 2013). In addition to that, TRAIL pathway activation was established as cancer-specific as TIC10 is able to induce apoptosis in cancer cells without demonstrating the same level of toxicity against normal cells (Greer and Lipkowitz 2015; Kline et al. 2018). TIC10 is currently under investigation in several phase I and phase II clinical trials for the treatment of cancer (Greer and Lipkowitz 2015) including many solid tumor entities as well as hematological malignancies in advanced stages.

3.2.3 PBI-05204 (Oleandrin)

PBI-05204 is derived from *Nerium oleander* and shows inhibitor properties against the α-3 subunit of Na–K ATPase, FGF-2 export, AKT, and p70S6K (Hong et al. 2014). The drug is currently examined in a phase I clinical trial for the treatment of advanced solid cancers regardless of cancer type (NCT00554268) and in a phase II clinical trial for treatment of metastatic pancreatic adenocarcinoma (NCT02329717). Interestingly, a study by Sreenivasan et al. (2003) suggested that PBI-05204 does not induce apoptosis in primary human cells and therefore may selectively inhibit malignant cells with elevated activation levels of target proteins the drug is directed at (Newman and Yang 2015).

3.2.4 Miltefosine and Perifosine (KRX-0401)

Miltefosine and perifosine (also referred to as KRX-0401), are alkylphosphocholine drugs that interact with the binding of several proteins to the plasma membrane, including AKT, by interacting with its PH2 domain (Gills and Dennis 2009). Miltefosine is the only FDA-approved AKT inhibitor on the market but is not approved for cancer therapy yet (Sunyoto et al. 2018). The approval is limited to the treatment of leishmaniasis (Berman 2015). Miltefosine has been examined in a phase II clinical trial (NCT02366884) together with other antibacterial, antifungal, and antiprotozoal agents for cancer treatment in the context of "atavistic chemotherapy". The theory implies that malignant cells may return to an ancestral primordial cell type and therefore are similar to unicellular organisms which include bacteria, yeast, and protozoal cells.

Perifosine was examined in a phase III trial for the treatment of multiple myeloma (NCT01002248) that was terminated (The Myeloma Beacon 2013) due to lack of extension of progression-free survival compared to bortezomib and dexamethasone alone. Another phase III trial was conducted using perifosine and capecitabine in refractory advanced colorectal carcinoma (CRC, NCT01097018). Although the phase II study with capecitabine plus perifosine in CRC patients detected an improved overall survival and a longer time to progression compared to capecitabine monotherapy (Richardson et al. 2012), the phase III study in patients treated with

the combination therapy showed no benefit of adding perifosine to chemotherapy in advanced CRC patients (Bendell et al. 2012).

3.3 AKT1 Specific Inhibitors

Differential roles of AKT isoforms have been identified in the past, especially in tumor cells (Hinz and Jucker 2019). Recent reports suggest a stimulating role of activated AKT1 on proliferation and survival of cancer cells including breast and ovarian cancer (Hinz and Jucker 2019; Linnerth-Petrik et al. 2016). Thus, selective inhibition of AKT1 may reduce commonly adverse events and unwanted off-target effects of pan-AKT inhibition. The differential roles identified for individual AKT isoforms underline the idea that pan-AKT inhibition does not always lead to success in cancer treatment despite its effect on the PI3K/AKT/mTOR pathway, which has pro-migratory and proliferative properties (Manning and Toker 2017). AKT isoforms commonly exhibit contradictory roles as, for example, increased migration and metastasis in a triple-negative breast cancer cell xenograft model after injection of MDA-MB-231 AKT3 knockdown cells (Grottke et al. 2016). These data suggest individual roles of AKT isoforms in different cancer entities that should be further examined and be considered in further drug development.

3.3.1 A-674563

A-674563 is a selective AKT1 inhibitor that acts in an ATP-competitive way. The drug was developed in 2005 and despite promising results from in vitro and in vivo studies in xenograft mouse models using cancer cell lines (Luo et al. 2005), the inhibitor never entered clinical trials.

However, the idea of selective inhibition of AKT isoforms may be used as a drug target. In NSCLC, opposing roles of AKT1 and AKT2 have been proposed. While AKT1 ablation decreases tumor growth, AKT2 deficiency accelerates tumor growth in an NSCLC xenograft mouse model (Linnerth-Petrik et al. 2014). Chorner and Moorehead examined the issue of differential functions of AKT isoforms and examined the function of the AKT1-specific inhibitor A-674563 in six NSCLC cell lines. The authors revealed a lower IC_{50} of A-674563 compared to treatment with the pan-AKT inhibitor MK-2206 and therefore concluded that AKT1 specific treatment is superior to pan-AKT inhibition in NSCLC (Chorner and Moorehead 2018).

3.3.2 RX-0201

Exclusive targeting of AKT1 was achieved by Yoon et al. who designed an antisense oligonucleotide (AO) with sequence complementarity to AKT1 mRNA (Yoon et al.

2009). AKT1 specificity was confirmed in preclinical models by western blot analysis of AKT1 expression and detection of AKT1 mRNA by RT-PCR. This AKT1 AO in combination with chemotherapeutic agents enhanced treatment response in a xenograft mouse model after subcutaneous injection of U251 human glioblastoma cells and MIA human pancreatic cancer cells in vivo (Yoon et al. 2009). Selective targeting of AKT1, but not AKT2 or AKT3, therefore may contribute to personalized treatment alternatives for different patients and different entities of cancer. RX-0201 has been examined in two phase II clinical trials for the treatment of metastatic pancreatic cancer (NCT01028495) and metastatic renal cell carcinoma (NCT02089334). In the phase II study in renal cell carcinoma patients, the combination of RX-0201 and everolimus was tolerable, safe, and exhibited promising efficacy in progression-free survival (Agarwal et al. 2019).

3.4 AKT2 Specific Inhibitors

3.4.1 CCT128930

CCT128930 was presented as a potent ATP-competitive inhibitor of AKT2 with the ability to reduce tumor growth in the BT474 breast cancer cell line and the U87MG human glioblastoma cell line in vitro as well as in a xenograft model in vivo (Yap et al. 2011). Although the drug demonstrated strong anti-tumor properties and was able to decrease activation of several downstream substrates of AKT including GSK3ß (Yap et al. 2011), the drug never entered clinical trials as a potential cancer therapy.

3.5 AKT1/2 Specific Inhibitors

3.5.1 AKT Inhibitor VIII

Lindsley et al. already addressed the lack of AKT-specific inhibitors in 2005 and developed an allosteric dual AKT1/AKT2 inhibitor (Lindsley et al. 2005). The authors reported that dual inhibition of AKT1 and AKT2 maximizes the frequency of apoptotic cells in various cancer cell lines including the ovarian cancer cell line A2780 and the prostate cancer cell line LNCaP. The inhibitors were additionally tested for their potency against particular AKT isoforms in an in vivo mouse model. The authors reported diminished levels of phosphorylated AKT1 and AKT2, but not AKT3, in mouse lungs after intravenous injection of their compound. (Lindsley et al. 2005). However, the results of dual inhibition of AKT1 and AKT2 are limited to preclinical work.

3.5.2 BAY1125976

BAY1125976 selectively binds to AKT1 and AKT2 and inhibits its activation in a non-ATP-competitive manner. In vitro experiments as well as in xenograft mouse models in vivo with breast cancer cells (KPL-4 and MCF-7), harboring activating PI3K/AKT/mTOR mutations or luminal B status revealed strong synergistic effect of BAY1125976 on proliferation when applied in combination with anti-hormonal treatment (Politz et al. 2016, 2017). Therefore, the authors concluded that the addition of BAY1125976 may be useful for the treatment of hormone-receptor-positive breast cancer (Politz et al. 2016). The drug entered a phase I clinical trial in 2013 (NCT01915576) and was completed in 2016. The results indicated that clinical benefit of BAY1125976 did not correlate with AKT status and was only able to induce a partial response in 1% of treated patients, whereas 38% had a stable disease and 49% a progressive disease according to the RECIST guidelines (Schneeweiss et al. 2019).

3.6 AKT3 Specific Inhibitors

To our knowledge, no specific inhibitors of AKT3 for cancer therapy have been established either in preclinical or in clinical work. AKT3 inhibition can mostly be observed as a result of pan-AKT inhibition (Stottrup et al. 2016).

3.7 Adverse Events Following AKT Inhibition

As AKT is part of the PI3K/AKT/mTOR signaling pathway, which regulates pivotal cellular processes (Osaki et al. 2004), inhibition leads to a not to be neglected number of adverse events which are often dose-limiting in clinical trials. Common adverse events of AKT inhibition include fatigue, rash, nausea and vomiting, diarrhea, glucose abnormalities, and weight loss (Xing et al. 2019). Dose-limiting toxicities in clinical trials are mostly due to gastrointestinal toxicity (Giantonio et al. 2004; Xing et al. 2019).

In addition to the side effects that are induced by the direct inhibition of AKT, also off-target effects and cross-reaction with other proteins and pathways have to be kept in mind. For example, AKT inhibitor TIC10, which inhibits AKT by TRAIL induction, additionally inhibits ERK, which belongs to the RAS/RAF/MEK/ERK pathway (Allen et al. 2013).

Table 1 Overview of AKT inhibitors for cancer treatment examined in preclinical or clinical settings

Drug/Compound	AKT specificity	Status	Reference	Clinical Trial ID
A-674563	AKT1 (ATP-competitive inhibitor)	No clinical trials	Chorner and Moorehead (2018)	n/a
AKT inhibitor VIII	AKT1 and AKT2	No clinical trials	Lindsley et al. (2005)	n/a
BAY1125976	AKT1 and AKT2 (allosteric inhibitor)	Phase I	Politz et al. (2017)	NCT01915576
RX-0201	AKT1, antisense oligonucleotide	Phase I and Phase II	Yoon et al. (2009)	NCT02089334, NCT01028495
CCT128930	AKT2, ATP-competitive inhibitor	No clinical trials	Yap et al. (2011)	n/a
Miltefosin	pan-AKT, allosteric inhibitor interfering with membrane binding	Phase II, FDA approval 2014*	Smorenburg et al. (2000)	NCT02366884

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(continued)

Drug/Compound	AKT specificity	Status	Reference	Clinical Trial ID
MK-2206	pan-AKT, allosteric inhibitor	Phase I, Phase II	Hirai et al. (2010)	NCT01147211, NCT01333475, NCT01186705, NCT01249105 NCT01240941, NCT01169649, NCT00670488, NCT01283035 NCT01240928, NCT01604772, NCT01253447, NCT01480154 NCT01370070, NCT01071018, NCT01319539, NCT01349933 NCT01307631, NCT01258998, NCT01802320, NCT01705340 NCT01239355, NCT00963547, NCT01277757, NCT01481129 NCT01466868, NCT01231919, NCT01245205, NCT01281163 NCT01294306, NCT01260701, NCT01776008, NCT01425879 NCT01783171, NCT00848718, NCT01344031, NCT01519427 NCT01235897, NCT01021748, NCT01239342, NCT01859182 NCT01263145, NCT01369849, NCT01295632, NCT01251861 NCT01658943, NCT01243762, NCT01714128, NCT01306045, NCT01248247, NCT01042379
Perifosine (KRX-0401)	pan-AKT, allosteric inhibitor interfering with membrane binding	Phase I, Phase II	Gills and Dennis (2009; Richardson et al. (2012)	NCT01224730, NCT01048580, NCT01049841, NCT00389077 NCT00873457, NCT00455559, NCT00448721, NCT00375791 NCT00590954, NCT01002248, NCT00498966, NCT00847366 NCT00398710, NCT00398879, NCT00776867, NCT00399789 NCT00391560, NCT00401011, NCT01051557, NCT00401388 NCT00422656, NCT00399152, NCT00398814, NCT00058214 NCT02238496, NCT00398697, NCT00301938, NCT00399087 NCT00399126, NCT00415064, NCT00064324, NCT00431054 NCT00062387, NCT01097018, NCT00005794, NCT00059982 NCT00060437, NCT00053924, NCT00054145, NCT00019656 NCT00053781, NCT00053794, NCT01555281, NCT00055380
Triciribine (also referred to as AKT inhibitor V, PTX-200, API-2)	pan-AKT (ATP-competitive inhibitor)	Phase I, Phase II	Schram and Townsend (1971)	NCT02930109, NCT01697293, NCT00642031, NCT00363454 NCT01690468

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Drug/Compound	AKT specificity	Status	Reference	Clinical Trial ID
GSK2141795 (Uprosertib)	pan-AKT (ATP-competitive inhibitor)	Phase I, Phase II	Dumble et al. (2014)	NCT01958112, NCT01941927, NCT01907815, NCT00920257 NCT01266954, NCT01902173, NCT01964924, NCT01989598 NCT01979523, NCT01935973, NCT01138085, NCT02093546
GSK690693	pan-AKT (ATP-competitive inhibitor)	Phase I	Rhodes et al. (2008)	NCT00666081, NCT00493818
LY2780301	pan-AKT (ATP-competitive inhibitor)	Phase I, Phase II	Azaro et al. (2015)	NCT01980277, NCT02018874, NCT01115751
ARQ 092 (Miransertib)	pan-AKT, allosteric inhibitor	Phase I	Harb et al. (2015)	NCT01473095
ARQ 751	pan-AKT, allosteric inhibitor	Phase I	Yu et al. (2015)	NCT02761694
AT13148	pan-AKT, ATP-competitive multi ACG kinase inhibitor	Phase I	Yap et al. (2012)	NCT01585701
AT7867	pan-AKT, ATP-competitive inhibitor	No clinical trials	Grimshaw et al. (2010)	n/a

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Drug/Compound	AKT specificity	Status	Reference	Clinical Trial ID
AZD5363 (Capivasertib)	pan-AKT, ATP-competitive inhibitor	Phase II, Phase II, Phase III	Davies et al. (2012)	NCT03772561, NCT02338622, NCT03310541, NCT02121639, NCT02077569, NCT01353781, NCT01895946, NCT04439123, NCT01226316, NCT02451956, NCT01992952, NCT02525068, NCT01692262, NCT02423603, NCT01625286, NCT04087174, NCT02208375, NCT02449655, NCT02576444, NCT03182634, NCT03742102, NCT02299999, NCT02117167, NCT02664935 NCT02465060, NCT03997123, NCT04493853, NCT04305496
GDC0068 (Ipatasertib)	pan-AKT, ATP-competitive inhibitor	Phase I, Phase II, Phase III	Lin et al. (2013)	NCT01562275, NCT02301988, NCT02536391, NCT04464174, NCT01362374, NCT02162719, NCT01896531, NCT03341884, NCT04060862, NCT02063581, NCT02390492, NCT01485861, NCT04434040, NCT04253561, NCT02430363, NCT03840200 NCT03959891, NCT03853707, NCT02465060
GSK2110183 (Afuresertib)	pan-AKT, ATP-competitive inhibitor	Phase I, Phase II	Dumble et al. (2014)	NCT01445587, NCT01532700, NCT01531894, NCT01395004 NCT01428492, NCT01653912, NCT01476137, NCT02040480 NCT00881946, NCT01827644
API-1	pan-AKT, prevents binding of AKT to plasma membrane	No clinical trials	Kim et al. (2010)	n/a

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Drug/Compound	AKT specificity	Status	Reference	Clinical Trial ID
ONC201 (TIC10)	pan-AKT, induces tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) inhibiting AKT and ERK	Phase I, Phase II	Allen et al. (2013)	NCT02038699, NCT02250781, NCT03733119, NCT02324621 NCT03034200, NCT04055649, NCT02420795, NCT02863991 NCT03791398, NCT03932643, NCT03099499, NCT03295396 NCT02609230, NCT03394027, NCT03416530, NCT02392572 NCT03485729, NCT03492138, NCT0313413
PBI-05204 (Oleandrin)	pan-AKT, nerium oleander extract that inhibits α-3 subunit of Na–K ATPase, as well as FGF-2 export, AKT, NF-κB, and p70S6K	Phase I, Phase II	Hong et al. (2014)	NCT02329717, NCT00554268

* Miltefosine is the only FDA-approved AKT inhibitor. Nevertheless, the approval is restricted to the treatment of cutaneous, mucosal, and visceral leishmaniasis (Sundar and Singh 2016). To date, no AKT inhibitor has been approved by the FDA for cancer therapy.

4 Immune Evasion

In addition to uncontrolled growth due to the accumulation of oncogenic mutations within crucial signaling pathways and loss of tumor suppressor genes, cancer cells also tend to increase their cellular survival (Hanahan and Weinberg 2000). Cellular survival of cancer cells is mediated by signaling pathways such as the PI3K/AKT/mTOR signaling pathway but also depends on immune evasion. Evasion of immune destruction has been recently established as a novel hallmark of cancer by Hanahan and Weinberg (Hanahan and Weinberg 2011).

Currently, treatment of cancer does not solely focus on cytotoxic drugs (e.g., chemotherapeutics) but also includes a wide range of targeted treatment options. In most cases, cytotoxic drugs unspecifically interfere with the DNA structure and cause damage to it in rapidly dividing cancer cells as well as in normal cells (Woods and Turchi 2013). In contrast, targeted treatments aim to selectively inhibit overactivated molecules or pathways and therefore lead to less adverse events (Baldo and Pham 2013). However, as these drugs are highly specific for their respective target and cancers usually accumulate several mutations during their establishment (Hanahan and Weinberg 2011), the combination with conventional chemotherapeutics is often used. Especially in malignant melanoma treatment, immune checkpoint inhibitors have gained tremendous attention over the last years after approval of CTLA-4 antagonist Ipilimumab (Ott et al. 2013). Nowadays, immune checkpoint inhibition approaches are widely used for other types of cancer besides melanoma (Vaddepally et al. 2020). Current FDA-approved targets for immune checkpoint inhibition include CTLA-4, PD1, and PD-L1 (Vaddepally et al. 2020). Using PD1 or PD-L1 antibodies, disruption of the inhibitory T cell signaling mediated by PD1/PD-L1 interaction can be prevented to overcome tumor immune escape mechanisms (Seidel et al. 2018).

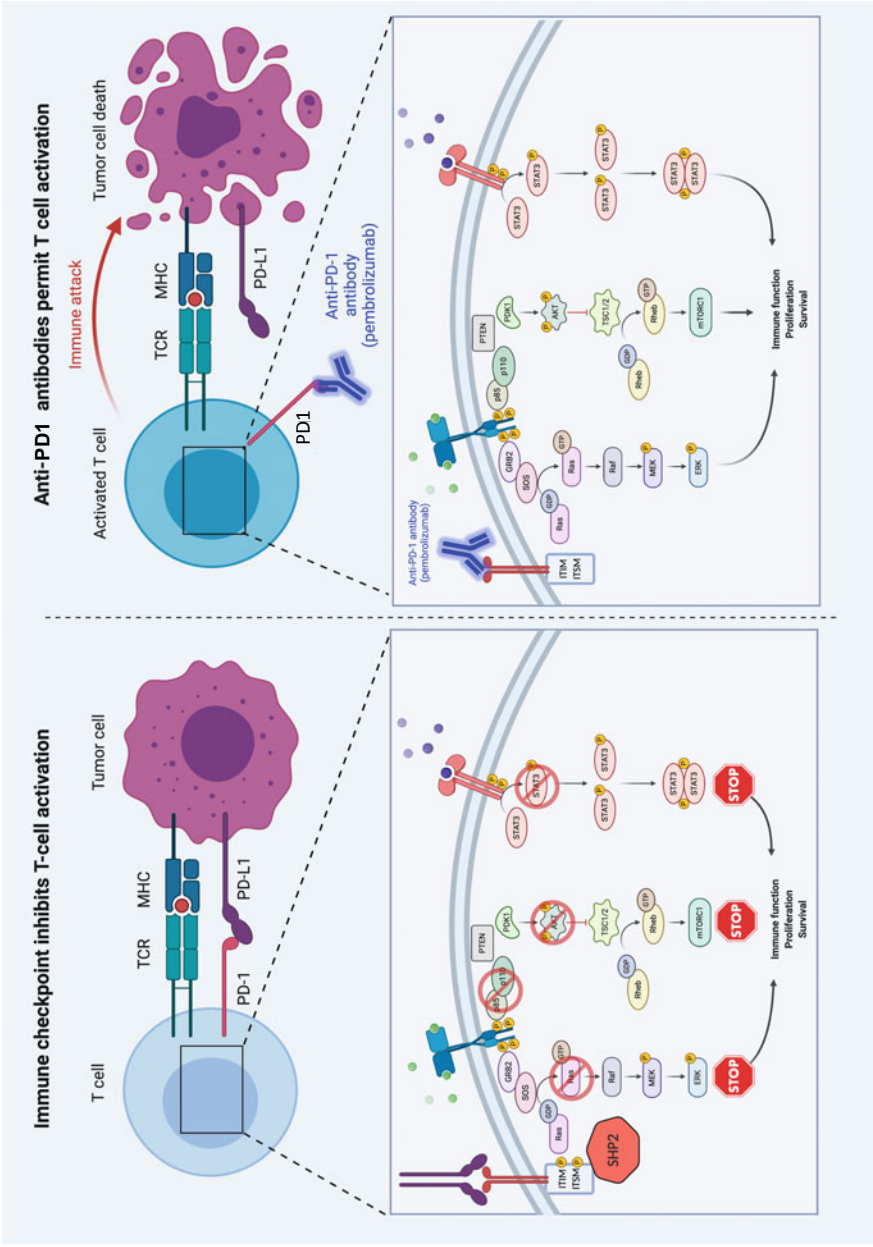
4.1 AKT Involvement in the Efficacy of Immune Checkpoint Inhibitors

The innate and adaptive arms of the immune system play a pivotal role in preventing infection, but they are not limited to recognizing foreign invaders. Although tumor cells develop upon mutations of endogenous cells and are thereby included in the self-tolerance mechanisms (Chaplin 2010) of the immune system, tumor-associated properties including necrosis and dysregulated growth, however, attract immune cells to the inflammatory tumor microenvironment (Gonzalez et al. 2018). Tumor cells expressing PD-L1 can prevent T cell activation by binding to PD1 expressed on the T cell surface. Activation of PD1 signaling leads to phosphorylation of ITIM and ITSM motifs of PD1 and therefore SHP2 recruitment. SHP2 is a phosphatase that catalyzes the dephosphorylation at tyrosine residues of a vast amount of growth- and effector function-inducing proteins that belong to the PI3K/AKT/mTOR,

RAS/RAF/MEK/ERK, and JAK/STAT pathways. Ultimately, dephosphorylation of upstream elements including PI3K results in decreased activation of AKT and therefore inhibition of the Bcl-xL-mediated anti-apoptotic effect (Wu et al. 2020). Physiologically, PD-L1 is expressed in cells of the innate and adaptive immune system and mediates T cell homeostasis and T cell tolerance by attenuation of the immune response (Chinai et al. 2015; Ostrand-Rosenberg et al. 2014).

Activation of T cells into an effector state relies on two juxtracrine signals, which include TCR-antigen binding and costimulatory signaling through CD28 and CD80/CD86 (also referred to as B7-1 and B7-2) ((Lenschow et al. 1996). Interestingly, in T cell signaling, costimulatory as well as coinhibitory signals are both mediated by CD80/CD86 dependent on the binding partner. One example of a coinhibitory signal is CTLA-4 (also known as CD152) which functions as an immune checkpoint by downregulating the immune response (Waterhouse et al. 1995). While CD28-CD80/CD86 binding stimulates T cell effector function, CTLA-4-CD80/CD86 binding inhibits T cell activation and induces cell cycle arrest in T cells by activating the serine-threonine phosphatase PP2A. Substrates of PP2A include AKT and MEK (Ruvolo 2016). Dephosphorylation of AKT, therefore, prevents the downstream signaling necessary for full T cell activation and effector function (Arasanz et al. 2017). CTLA-4 antagonists compete with CD80/86 for CD28 binding but have a higher affinity than CD80/86 and therefore diminish the inhibitory effect on the immune system and enhance adaptive immune cell effector function (Qin et al. 2019). Hence, the occupation of CTLA-4 receptor decreases the binding frequency of CTLA-4 with CD80/86 and increases CD28-CD80/86 binding (Chambers et al. 2001), which leads to the observed anti-tumor effects.

The anti-CTLA-4 antibody Ipilimumab was the first approved immune checkpoint inhibitor for the treatment of malignant melanoma, and since then many drugs that target CTLA-4 have successfully entered phase III and phase IV clinical trials (Darvin et al. 2018). Recently, the FDA extended the approval of PD1 antibody pembrolizumab (also referred to as MK3475) (Kwok et al. 2016), initially developed for melanoma treatment, to all solid tumor entities (Merck & Co. Inc. 2020). Therefore, immune checkpoint inhibition opens up a new avenue of cancer therapy in patients that progressed on prior treatment or in cancer types that do not have other satisfactory treatment options (Merck & Co. Inc. 2020) (Fig. 1).


Anti-PD1 antibodies permit T cell activation
Immune attack
Tumor cell death
Activated T cell
TCR
MHC
PD-L1
PD1
Anti-PD-1 antibody (pembrolizumab)
Immune checkpoint inhibits T-cell activation
T cell
Tumor cell
PD-1
SHP2
STOP
Immune function
Proliferation
Survival

◂**Fig. 1 PD1 signaling inhibits T cell activation through inactivation of PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and STAT signaling as part of tumor immune escape** In order to protect the host against foreign intruders, there is a continuous immune surveillance by the innate and adaptive immune system. To maintain the balance between immune tolerance and immune effector functions, regulatory mechanisms, considered as immune checkpoints, have been identified in the past.

Tumor cells can express cell surface markers that inhibit T cell activation and proliferation, including PD-L1, therefore escaping the immune effector cells' response. T cell activation relies on three signals including binding of the T cell receptor to MHC bound to an antigen, costimulatory signals (e.g., CD80/CD86, CD70, CD137, ICOS, 4-1BB, and OX40), and cytokine release either through paracrine secretion from other immune cells (e.g., TNFs, ILs, IFNs) or autocrine secretion (e.g., IL-2).

For full T cell activation and acquisition of effector functions, signaling through intracellular pathways including PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and JAK/STAT is required. Tumor cells expressing PD-L1 on their surface can negatively affect T cell survival and proliferation through PD1 signaling. Upon binding of PD-L1 to PD1 receptor expressed on the surface of a T cell, SHP2 catalyzes the dephosphorylation of key molecules of the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK signaling pathways. Treatment with PD1 antibodies can block PD1 receptors and therefore prevent suppression of AKT signaling leading to increased effector functions, survival, and proliferation of T cells.

5 Conclusions

Constitutive activation of signal transduction pathways is considered as a hallmark of cancer (Hanahan and Weinberg 2011) and results in increased tumor growth. The PI3K/AKT/mTOR pathway, which mediates cell growth, utilization of nutrients, and other crucial cellular processes, is one of the most commonly hyperactivated pathways in cancer (Osaki et al. 2004). AKT is a key molecule of this pathway and also establishes crosstalk with other signal transduction pathways (Janku et al. 2018). Development of AKT inhibitors is associated with a high degree of complexity due to the considerable number of feedback loops that AKT is part of, crosstalk with other pathways, and the vast number of AKT substrates (Janku et al. 2018). Currently, despite the promising effect of AKT inhibition in preclinical work, no AKT inhibitor has entered phase IV clinical trials yet nor is approved by the FDA for cancer treatment.

Immune evasion and immunotherapy became emerging topics over the last years, especially in tumor entities including malignant melanoma, with low response rates to conventional anti-tumor therapies (Ott et al. 2013). Although immune checkpoint inhibitors target surface molecules including PD1, PD-L1, or CTLA-4 (Ott et al. 2013), the intracellular signal transduction relies on intracellular AKT signaling (Arasanz et al. 2017; Wu et al. 2020). Therefore, AKT is not only a direct target that can be inhibited directly through AKT inhibitors in cancer therapy but also plays a key role in the efficacy of immune checkpoint inhibitors as their signaling relies on the PI3K/AKT/mTOR pathway.

The most promising candidate of all AKT inhibitors in clinical trials is Ipatasertib (Saura et al. 2017) which is currently being examined in a phase III clinical trial for the treatment of locally advanced or metastatic prostate cancer (NCT03072238) and breast cancer (NCT04060862, NCT04177108, NCT03337724). Pan-AKT, ATP-competitive inhibitors, to which Ipatasertib belongs, are the majority of all AKT inhibitors tested. However isoform-specific inhibitors have also been tested with reduced adverse events. Although AKT isoforms share a high homology, differential tissue distribution, functional roles and impact on prognosis have been identified in the past that may lead a step ahead to a personalized, mutational status-based, cancer therapy.

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Developing PI3K Inhibitors for Respiratory Diseases

E. Fagone, M. Fruciano, E. Gili, G. Sambataro, and Carlo Vancheri

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E. Fagone · M. Fruciano · E. Gili · G. Sambataro · C. Vancheri (✉)
Department of Clinical and Experimental Medicine, Regional Referral Center for Rare Lung Diseases, University Hospital "Policlinico G. Rodolico-San Marco", University of Catania, Catania, Italy
e-mail: vancheri@unict.it

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Abstract A number of different experimental models using both non-selective and selective PI3K inhibitors have shown that many pathogenic steps of respiratory disorders, such as bronchial asthma, Chronic Obstructive Pulmonary Disease (COPD), Idiopathic Pulmonary Fibrosis (IPF), Acute Respiratory Distress Syndrome (ARDS) and Lung Cancer (LC) are, at least in part, regulated by the PI3K signaling pathway, suggesting that the inhibition of PI3K could represent an ideal therapeutic target for the treatment of respiratory diseases. This chapter summarizes the current state of the therapeutic strategies aimed to exploit the inhibition of PI3K in this context. In animal models of asthma, selective δ and γ inhibitors have shown to be effective, and when administered by inhalation, reasonably safe. Nevertheless, very few clinical trials have been performed so far. The efficacy of current traditional therapies for allergic bronchial asthma has likely diminished the need for new alternative treatments. Surprisingly, in COPD, where instead there is an urgent need for new and more effective therapeutic approaches, the number of clinical studies is still low and not capable yet, with the exception for an acceptable safety profile, to show a significant improvement of clinical outcomes. In IPF, a disease with a disappointing prognosis, PI3K inhibitors have been bound to a FAP ligand with the aim to selectively target myofibroblasts, showing to significantly reduce collagen production and the development of lung fibrosis in an animal model of lung fibrosis. Due to its role in cell activation and cell replication, the PI3K pathway is obviously largely involved in lung cancer. Several studies, currently ongoing, are testing the effect of PI3K inhibitors mainly in NSCLC. Some evidence in the treatment of cancer patients suggests the possibility that PI3K inhibitors may enhance the response to conventional treatment. The involvement of PI3Kδ in the modulation of airway neutrophil recruitment and bronchial epithelial functional alterations also suggest a potential role in the treatment of ARDS, but at the current state the ongoing trials are aimed to the treatment of ARDS in COVID-19 patients. In general, few clinical trials investigating PI3K inhibitors in respiratory disorders have been performed so far. This relatively new approach of treatment is just at its beginning and certainly needs further efforts and additional studies.

1 Introduction

In any disease, the development of specific and effective medical treatments is strictly associated to the understanding of its pathogenic mechanism. Respiratory diseases such as bronchial asthma, Chronic Obstructive Pulmonary Disease (COPD), Idiopathic Pulmonary Fibrosis (IPF), lung cancer, and Acute Respiratory Distress Syndrome (ARDS) are no exception to this rule. In general, these clinical conditions are characterized by the activation, differentiation, proliferation, and persistence into the bronchi and/or lung tissue of inflammatory, immune and resident cells that triggered by specific risk factors (e.g., allergens, smoking, environmental exposures) may lead to different degrees of inflammation, fibrotic damage, and in some cases to the burgeoning of cancer. In these circumstances, several cellular signaling cascades

are abnormally activated and many chemical and molecular mediators are released. Recent evidence has demonstrated the role of intracellular kinases such as the lipid kinase phosphatidylinositol-3-kinase (PI3K) in the pathogenesis of a large spectrum of disorders including respiratory diseases. Based on their structure, distinct phospholipid substrates and different regulatory activity, PI3K is divided into classes I, II, and III. Class I is further divided into two subclasses: subclass IA, which includes the isoforms p110α, p110β, and p110δ, and subclass IB which is only composed of the isoform p110γ (Cantley 2002; Bi et al. 2002). Increased activity or mutations of PI3K results in an enhancement of phosphorylated protein kinases such as AKT that is strictly associated with the pathogenesis of different cancers including lung cancer (Samuels and Ericson 2006). PI3Kβ has instead been involved in the pathogenesis of colon cancer and its main activity is related to the regulation of the formation of blood clots. PI3Kδ is expressed by circulating leukocytes (mast cells, neutrophils, eosinophils, T lymphocytes) and endothelial cells. In a similar manner, PI3Kγ expression is largely restricted to immune cells, although some expression has been also detected in structural cell populations such as fibroblasts, and smooth muscle cells (Chan et al. 2021). Therefore, PI3Kδ and PI3Kγ are particularly involved in respiratory diseases such as asthma or COPD, characterized in their pathogenesis, by the participation of immune and inflammatory cells.

The role played by the PI3K family in the biological modulation of crucial cellular functions entails that any dysfunction of this regulatory activity could be associated with a state of disease, suggesting that the inhibition of PI3K could represent an ideal therapeutic target for the treatment of different diseases (Manning and Cantley 2007). Recent studies have highlighted that the PI3K pathway is also dysregulated in different lung pathologies, spanning from those characterized by inflammation and/or fibrosis to cancer (Vivanco and Sawyers 2002; Janku et al. 2018).

In this chapter, the role of the PI3K signaling pathway in the pathogenesis of different lung diseases will be reviewed with special attention to the more recent evidence of pharmacological approaches based on the inhibition of this pathway.

2 PI3K and Bronchial Asthma

2.1 Background

It has been extensively demonstrated that an imbalance between Th1 and Th2 lymphocytes in favor of Th2 cytokines such as IL-4, IL-5, and IL-13 is crucial for allergen-induced B cell production of IgE and for the recruitment of eosinophils, both crucial steps in the pathogenesis of allergic bronchial asthma (Galli and Tsai 2008). Airway inflammation is initially triggered by the interaction between allergen-specific IgE and mast cells. The subsequent activation of mast cells causes the expression and release of mediators and cytokines that promote airway bronchoconstriction, mucus overproduction, bronchial hyperresponsiveness, and amplify the synthesis of

immunoglobulin E (IgE). Furthermore, the activation of eosinophils, through the release of other inflammatory mediators, is responsible for chronic airway inflammation and bronchial remodeling, quintessential features of asthma (Hakim-Rad et al. 2009; Boyce et al. 2009).

2.2 *Are the Expression and Functional Profile of PI3K Relevant to the Pathogenesis of Bronchial Asthma?*

Several in vitro and in vivo studies have suggested that the expression and functional profile of PI3K is relevant for the pathogenesis of allergic bronchial asthma (Ito et al. 2007). Most of the evidence produced has used both non-selective and selective inhibitors of PI3K that have indirectly demonstrated the fundamental role of PI3K in many pathogenic aspects of bronchial asthma. Kämpe et al. (2012) investigated the role of PI3K in regulating eosinophil and neutrophil degranulation in allergic rhinitis and asthma. These cells were incubated with wortmannin, a non-selective PI3K inhibitor, and then stimulated with C3b-coated sephadex particles. Wortmannin reduced in a dose-dependent way the release of eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and myeloperoxidase. However, this effect was lower in cells derived from allergic asthmatic patients during pollen season or after bronchial challenge, suggesting that in these conditions, in addition to PI3K, other signaling pathways might be involved. Other PI3K class I pan-inhibitors including CL27c, LY294002, and the same wortmannin have been shown to reduce eosinophil, neutrophil, and lymphocyte activity and recruitment in various experimental murine models of asthma or allergic inflammation. CL27c showed anti-inflammatory effects in a murine acute model of allergic asthma. This prodrug, which can be administered by inhalation, decreases leukocyte infiltration into the lungs, reduces the number of inflammatory cells in broncho-alveolar lavage (BAL) as well as the production of inflammatory mediators, and most importantly, it exerts a significant improvement of lung function. This study is particularly important for the route of administration that has been used to treat animals. Local treatment may have the advantage of allowing the optimization of the therapeutic efficacy, but at the same time, it could reduce the risk of unwanted adverse events that a systemic treatment could cause (Campa et al. 2018). Other authors have demonstrated that intratracheal administration of LY294002 in OVA-sensitized mice significantly reduces inflammatory cell counts and IL-5, IL-13, and CCL11 (eotaxin) levels in BAL. In addition, a marked reduction of eosinophil infiltration and mucus production was described into the peribronchial and perivascular connective tissue. This was functionally accompanied by a reduction of OVA-induced airway hyperresponsiveness to inhaled methacholine. Furthermore, western blot analysis of lung tissue derived from OVA-challenged animals demonstrated a marked reduction of the phosphorylation of AKT, downstream mediator of the PI3K pathway. (Duan et al. 2005; Marwick et al. 2010).

In the same OVA-induced asthma animal model, Kwak and coworkers have shown a significant increase in PI3K activity after allergen challenge and a parallel dramatic reduction of PTEN, known to counteract PI3K activity. They describe that the immunoreactivity for PTEN, localized in the bronchiolar epithelium in control mice, virtually disappeared in OVA-challenged lungs. Once again, the intratracheal administration of either wortmannin or adenoviruses carrying PTEN were capable of reducing IL-4, IL-5, and ECP levels in BAL as well as airway inflammation and bronchial hyperreactivity (Kwak et al. 2003).

2.3 Are PI3K Inhibitors Safe and Clinically Relevant in Allergic Bronchial Asthma?

Taken together, these studies provide further support to the hypothesis that the PI3K pathway plays a major role in the pathogenic mechanisms underlying asthma, suggesting that the inhibition of PI3K could represent an ideal approach for the treatment of asthma (Ito et al. 2007; Duan et al. 2005; Yoo et al. 2017). This observation, although very interesting and apparently promising, has raised many concerns on the use of non-selective inhibitors for therapeutic purposes. PI3K pan-inhibitors, blocking all PI3K isoforms, may cause systemic adverse events precluding their clinical use. New isoform-selective PI3K inhibitors, expected to be less toxic, have been already developed with the aim of representing more specific and effective inhibitors and, at the same time, safer molecules. This is largely based on a growing number of reports showing a specific involvement of isoforms γ and δ in airway inflammation and asthma. Indeed, several works have shown that PI3K isoforms γ and δ are upregulated during lung inflammation and airway remodeling, suggesting a major role for these two isoforms in the pathogenesis of asthma (Fruman and Bismuth 2009; Kok et al. 2009; Rommel et al. 2007).

In 2003, the first PI3Kγ-selective inhibitor, a 5-phenylthiazole derivative, was eventually developed, opening a new, possible therapeutic approach for respiratory disorders marked by airway inflammation. From then on, a variety of single or combined selective-PI3Kγ-PI3Kδ inhibitors have been tested to assess their efficacy and safety in in vitro experimental settings first, then in animal models and more recently in human clinical trials (Bruce et al. 2003).

PI3Kγ is involved in the modulation of mast cell degranulation, ROS release, eosinophil recruitment, neutrophil activation, and Th2 cytokine production (Ghigo et al. 2010). Laffargue et al. (2002) have demonstrated that mast cells derived from PI3Kγ-deficient mice have a weaker degranulation and a reduced ability to respond to IgE receptor cross-linking. In addition, animals passively immunized and antigen-challenged were protected from anaphylaxis. Based on their results these authors hypothesize that PI3Kγ, through its activity on mast cells, has a crucial role in the modulation of allergic inflammation. Moreover, in the same animal model, Wymann et al. (2003) observed reduced eosinophil recruitment at 48 hours post-challenge

compared with control group, suggesting that PI3Kγ plays also a role in the maintenance of eosinophilic inflammation in vivo. It is worth reminding here that the permanence of eosinophils within the bronchial tissue is crucial for the establishment of two typical features of severe asthma, chronic airway inflammation and bronchial remodeling.

PI3Kδ is also involved in the early phases of activation and allergen-IgE-induced mast cell degranulation, maturation, and differentiation of Th1, Th2, Th17, Treg cells, B lymphocyte activation, and antibody production. Indeed, T cells isolated from transgenic mice characterized by the absence of PI3Kδ catalytic activity (p110δ D910A/D910A T cells) showed reduced Th1 and Th2 differentiation after stimulation in vitro with anti-CD3 and anti-CD28 (Hawkins and Stephens 2015; Okkenhaug et al. 2002).

Mutation of PI3Kδ also induces alterations in mast cells and neutrophils, in allergen-IgE-induced degranulation and cytokine release. Indeed, inactivation of PI3Kδ restores/protects anaphylactic allergic responses in vivo (Ali et al. 2008; Puri et al. 2004). In this respect, it has been shown that IC87114, a selective inhibitor of the δ isoform, reduces lung tissue eosinophils, leukotriene C4, mucin overproduction, release of IL-4, IL-5, IL-13, vascular cell adhesion molecule-1 (VCAM-1), Chemokine (C–C motif) ligand 5 (CCL5), and CCL11. The same compound is also able to reduce the production of IgE in an OVA animal model of asthma (Lee et al. 2006a).

One of the prevailing features of airway inflammation and bronchial asthma is mucosal edema, mainly due to an increase in vascular permeability. Lee et al. (2006b) have clearly shown that in the murine OVA model of asthma, IC87114, a selective PI3Kδ inhibitor, reduces OVA-induced airway infiltration of inflammatory cells, production of Th2 cytokines, airway hyperresponsiveness, and vascular permeability. Furthermore, the selective inhibition of PI3Kδ reduces the increase of vascular endothelial growth factor (VEGF) levels induced by OVA challenge. Based on these results the authors hypothesized that PI3Kδ, through the inhibition of VEGF, prevents vascular leakage and the formation of mucosal edema, thus diminishing airway inflammation.

In this model, PI3Kδ inhibitors such as IC87114 (El-Hashim et al. 2017) and INK654 (Kim et al. 2020), as well as a double-selective compound blocking both PI3Kγ and PI3Kδ named TG100-115, effectively attenuate allergic bronchial inflammation and airway hyperresponsiveness reducing eosinophils, IL-4, IL-5, and IL-13 levels, total serum IgE (Lee et al. 2006a), airway mucus hypersecretion and, most importantly, improved lung function (Doukas et al. 2009). In addition, IC87114, by blocking VEGF upregulation, decreases vascular permeability and extravascular remodeling, responsible for asthmatic submucosal edema in bronchial tissue (Lee et al. 2006b). Recently, Jing Bi et al. have hypothesized that glucocorticoid insensitivity, a main feature of severe asthma and COPD, might be also related to the regulatory activity of PI3K. In this in vitro study, they observed that peripheral blood mononuclear cells (PBMCs) derived from patients with severe asthma had an evident dose and time-dependent insensitivity to glucocorticoids compared to mild asthma or normal individuals, which was associated with a diminished activity

of histone deacetylase 2 (HDAC2) and elevated expression of pro-inflammatory genes such as subunits of the transcription factors NFκB and AP1. Interestingly, BEZ235 (Dactolisib) and LY294002, a dual γ/δ and a PI3K pan-inhibitor, respectively, improved glucocorticoid responsiveness in PBMCs of patients with severe asthma. The results presented by Bi et al. (2020). suggest that PI3K, and more specifically the γ/δ isoforms, could be directly involved in the lack of sensitivity of inflammatory cells to the activity of steroids which is one of the major clinical problems of severe asthma, but certainly also relevant for COPD. A reduction of HDAC activity, at least in COPD, has already been shown to be one of the main reasons for the steroid insensitivity that characterizes this disease. However, this intriguing study has the limitation of being an in vitro study and certainly needs to be confirmed by observations produced in vivo. During the last few years some interesting clinical studies have been performed, that investigated the efficacy of selective PI3K inhibitors in allergic rhinitis and allergic bronchial asthma. Idelalisib is a PI3Kδ inhibitor already approved for the treatment of some hematologic diseases with a recognized inhibitory activity toward allergen-induced activation of mast cells and basophil degranulation. In a phase 1 randomized, double-blind, placebo-controlled study, after 7 days of oral treatment with idelalisib nasal symptom score was registered in allergic rhinitis patients and compared to placebo group. After allergen challenge, idelalisib induced a significant improvement of symptoms, nasal airflow and nasal secretion. In this study, clinical data are consistent with the anti-inflammatory profile of idelalisib. Even so, authors of this study were very cautious on suggesting a regular use for idelalisib in allergic rhinitis, mainly because the study period was very short and did not consider the effect of a longer or chronic treatment as required in allergic rhinitis (Horak et al. 2016).

In a randomized, double-blind, placebo-controlled study, the efficacy and safety of inhaled nemiralisib (GSK2269557), another PI3Kδ inhibitor, were investigated in patients with uncontrolled asthma. The safety profile was good, with exception of some post-inhalation cough that was the most common side effect. After 14 days of treatment, nemiralisib reduced the levels of several pro-inflammatory cytokines such as IL-5, IL-6, IL-8, and IL-13 in sputum. Disappointingly, no significant improvement of lung function was observed. As stated by the authors of the study it is evident that a number of variables may have affected the result of this interesting trial including patient selection, sample size of the study, or duration of treatment (Khindri et al. 2018).

2.4 Closing Remarks

During the last few years, the role of PI3K in allergic bronchial asthma has been thoroughly investigated. Virtually all pathogenic steps of allergic bronchial asthma, from airway inflammation to bronchial remodeling are, at least in part, regulated by the PI3K signaling pathway. This was largely demonstrated by in vitro and preclinical experimental models using different non-selective and selective PI3K inhibitors. In

particular, selective δ and γ inhibitors have shown to be particularly effective, and when administered locally by inhalation, reasonably safe. Despite all these positive results, very few functional, clinical advantages have been described. In the face of a great number of in vitro and in vivo studies, very few clinical trials have been performed so far. Different reasons may explain, at least in part, this disappointing "scenario". Current traditional therapies for allergic bronchial asthma are in most of the cases very effective, determining a substantial lack for an urgent need of new alternative treatments. Thanks to the use of more selective PI3K inhibitors and the possibility to administer them by inhalation some safety issues may be solved. PI3K is certainly one of the main actors in the pathogenesis of asthma, but very likely not the only one, as often happens in biology, other redundant pathways may mimic or intervene when the PI3K pathway is blocked. Hopefully, this will be better investigated in the near future and new clinical trials examining the effect of PI3K inhibitors will be designed.

3 PI3K and Chronic Obstructive Pulmonary Disease (COPD)

3.1 Background

Chronic Obstructive Pulmonary Disease (COPD) is a condition characterized by chronic inflammation, emphysema, and fibrosis of small airways, leading to a slowly progressive, but irreversible lung damage with obstructive respiratory failure (Barnes et al. 2015). COPD represents one of the leading causes of death worldwide. It is associated with smoking or environmental and professional exposures; additional risk factors include age, low or high body mass index, low education level, and poverty (Osman et al. 2017). A genetic predisposition was supposed for the association of emphysema with some Mendelian syndromes as α1-antitripsin deficit, but more likely the genetic background should be assessed in response of the subject to smoke: no-smoking first-degree relatives of COPD patients have a similar risk of developing COPD as the general population, whereas smoking first-degree relatives had a three-fold increased risk (Barnes et al. 2015). Smoking and other "irritants" activate pattern recognition receptors, enabling an innate immune response characterized by an increased number of macrophages and neutrophils in the lungs. These cells show an impairment in phagocytosis with an incomplete clearance of bacteria and apoptotic cells, favoring bacterial colonization, chronic inflammation, and activation of airway epithelial cells and mucus secretion. The action of smoking on innate immune cells persists also after smoking cessation. In successive phases, adaptive immunity also plays a role, with an increased number of mast cells, B and Th1 lymphocytes, that amplify the process (Brusselle et al. 2011). The main pathogenic mechanism is related to oxidative stress, induced by the "irritating" action of smoking

on bronchial and alveolar cells. Reactive Oxygen Species (ROS) activate inflammatory genes and proteases through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and mitogen-activated protein kinase (MAPK) and inhibit endogenous antiproteases such as α1-antitrypsin. The increased proteolysis, together with the activation of B cells, generate autoantibodies able to increase inflammation and its persistence. ROS activates transforming growth factor beta (TGF-β) response with increasing fibrosis, causes DNA damage, also interfering with the mechanism of repairing, and decreases the activity of sirtuin-1, favoring an accelerated cellular aging (Barnes et al. 2015).

3.2 Are the Expression and Functional Profile of PI3K Relevant to the Pathogenesis of COPD?

Different signaling pathways such as the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway (Montero et al. 2021), TGF-β signaling pathway (TGF-β/Smad), MAPK pathway, NFκB, and PI3K are activated in COPD (Manley et al. 2019; Edwards et al. 2009; Moradi et al. 2021). Several studies have shown that PI3K class I isoforms and specifically PI3Kγ and δ, play a relevant role in the physiopathology of COPD. Although all class I PI3Ks are expressed in leukocytes, PI3Kγ and PI3Kδ are the main and specific mediators in innate and adaptive immune responses. Therefore, it is plausible that PI3K signaling may affect intercellular cross-talks between alveolar immune cells and airway epithelial cells in COPD (Wymann and Marone 2005; So and Fruman 2012). It has been described that PI3K and its downstream mediators are upregulated in primary human bronchial epithelial cells facilitating chronic airway inflammation, mucus secretion, and ultimately alveolar wall damage (Ito et al. 2007; Yoo et al. 2017). Moreover, lungs of COPD patients show exaggerated immune responses with high levels of monocyte infiltration, alveolar macrophage activation, and neutrophil migration due to an increased activation of PI3Kδ (Sriskantharajah et al. 2013). Expression of PI3Kδ and AKT phosphorylation is enhanced in COPD alveolar macrophages. It is oxidative stress that directly induces PI3Kδ-dependent AKT activation, leading to the loss of steroid sensitivity, which is one of the main clinical features of COPD (Barnes 2016). Both γ and δ isoforms, controlling the interactions between leukocytes and epithelial/endothelial cells also regulate chemotaxis and migration of infiltrating cells, such as neutrophils (Okkenhaug et al. 2002; Hirsch et al. 2000). Their activity has been demonstrated in various types of inflammatory cells as described by several experiments with knockout animals or with pan-PI3K or isoform-specific inhibitors (Fruman and Bismuth 2009; Kok et al. 2009; Rommel et al. 2007; Wymann and Schneiter 2008). Therefore, PI3K isoforms are considered promising therapeutic targets in the development of future treatments for inflammatory lung diseases such as COPD (Ghigo et al. 2010).

The PI3K class I pan-inhibitors, LY294002 and wortmannin, effectively reduce total lymphocyte counts and chemotaxis of neutrophils in vitro and in various

murine experimental models of COPD. In vitro studies show that LY294002 reduces neutrophil migratory speed, whereas wortmannin induces alveolar stem cell differentiation and alveolar repair in elastase-induced COPD or in cigarette smoke-exposed mice (Sapey et al. 2011). In this study, the alveolar repairing effect of wortmannin was evaluated in murine models of COPD using CT scans and histology. The radiologic score evaluated in terms of lung density (Hounsfield Units values) showed a complete recovery of animals treated with wortmannin. This was confirmed by histology and by the improvement in lung function suggesting a potential role for PI3K inhibition in COPD.

Different PI3K inhibitors, such as ZSTK474 and GSK045, have been studied in vitro by Gupta et al. (2016) for their inhibitory effects on the production of matrix metalloproteinase-9 (MMP-9) and extracellular reactive oxygen species (ROS) from cultured blood and sputum neutrophils isolated from COPD patients. Blood and sputum neutrophils derived from COPD patients were incubated with dexamethasone, a pan PI3K inhibitor (ZSTK474), or a selective PI3Kδ inhibitor (GSK045). After a period of incubation with the different drugs, neutrophil production of MMP-9 and ROS was measured. GSK045 significantly inhibited MMP-9 and ROS release from neutrophils derived from patients with either a stable disease or during a disease exacerbation. It is important to note that in the same model the effect of a MAPK inhibitor and that of dexamethasone were lower. Authors concluded that the selective inhibition of PI3Kδ could be "a potential strategy for targeting glucocorticoid insensitive MMP-9 and ROS secretion from COPD neutrophils".

Moreover, IC87114 and theophylline, selective PI3Kδ inhibitors, upregulate HDAC2 expression in vitro and restore steroid response in PBMC from COPD patients (To et al. 2010). Furthermore, Ford et al. (2010) have shown that theophylline increases HDAC2 expression and corticosteroid sensitivity both in vitro and in animal models of COPD, through a specific inhibition of PI3Kδ. PI3Kδ is known to be activated in COPD lungs by oxidative stress, for those selective inhibitors of PI3Kδ mimic the effects of theophylline in restoring corticosteroid sensitivity.

3.3 Are PI3K Inhibitors Safe and Clinically Relevant in COPD?

Some novel PI3Kδ inhibitors including LAS191954 and IHMT-PI3Kδ-372 have been recently evaluated for their activity in airway inflammation and COPD. LAS191954 was tested in a rat model of ConA-induced IL-2 production showing a significant reduction of T cell cytokine production. Feng Li et al. (2020). instead, concentrated their attention on IHMT-PI3Kδ-372, a newly discovered and potent PI3Kδ inhibitor. Inhaled IHMT-PI3Kδ-372 was administered to animals exposed to cigarette smoke; this animal model mimics the inflammatory changes observed in COPD. They described a dose-dependent improvement of lung function as well as an increase in arterial oxygen saturation (PaO_2) and a concomitant decrease of arterial carbon

dioxide ($PaCO_2$). Inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF as well as the number of alveolar macrophages in BAL were also reduced in a dose-dependent manner. The efficacy, at least in animal models, and the possibility to use IHMT-PI3Kδ-372 by inhalation, likely reducing the possibility of systemic side effects, makes this molecule a good candidate for further studies addressed to the treatment of COPD.

More recently, based on the multitude of experimental and preclinical data supporting the role of PI3K in COPD, the first clinical trials have been performed mainly aimed to evaluate primarily safety and then efficacy. Cahn et al. designed a randomized, double-blind, placebo-controlled study where GSK2265557 was given by inhalation once a day for 14 days, and during this period, possible side effects were recorded as well as cytokine levels in sputum and lung function. The most common adverse events were cough and headache, no other serious side effects were reported. Cytokine levels were reduced in sputum, but even so, airway resistance (Raw) and airway conductance were disappointingly not affected by the treatment. The authors conclude that their results encourage further studies aimed to explore the therapeutic role of PI3K in COPD. Another recent clinical trial evaluated the safety and efficacy of nemiralisib, a PI3Kδ inhibitor, in COPD patients during an acute exacerbation. It was a double-blind, placebo-controlled study. COPD patients having an exacerbation of the disease inhaled nemiralisib once daily for 12 weeks. During this period, safety profile, lung function, rate of new exacerbations, and rescue medication used, were recorded. The most common side effect was cough, particularly in patients receiving higher doses of the drug. Lung function, rate of new exacerbations, use of rescue medication were not statistically different in placebo- versus nemiralisib-treated groups (Fahy et al. 2021).

3.4 Closing Remarks

The possibility to exploit the PI3K pathway for the treatment of COPD has a solid scientific rationale. Based on a variety of evidence, although almost exclusively obtained by in vitro and animal model studies, the inhibition of this pathway can restore the sensitivity to corticosteroids, induce a reduction of airway inflammation and increase the alveolar repairing activity. Unfortunately, in none of the studies where respiratory functional parameters were part of the required outcomes, neither in the animal models nor in human studies, a significant improvement was described. Differently from bronchial asthma where medical treatments are very effective, in COPD there is an urgent need for new and radical approaches capable of changing the course of this devastating disease. Nevertheless, the number of clinical studies investigating the effect of PI3K inhibitors in the clinical context of COPD is surprisingly low. In the few studies performed so far, thanks also to the possibility of administering the PI3K inhibitors by inhalation, the safety profile has been considered acceptable and limited in most of the cases to some post-inhalation cough. Hopefully, in the

near future more clinical studies will explore in detail the opportunity to use PI3K inhibitors for the treatment of COPD.

4 PI3K and Idiopathic Pulmonary Fibrosis

4.1 Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, interstitial lung disease of unknown etiology with a median survival of 3–5 years from diagnosis. IPF is characterized by bronchiolar-alveolar epithelial-mesenchymal transition, fibroblast activation, excessive deposition of collagen and extracellular matrix, lung tissue fibrosis, and eventually by the decline of pulmonary function and respiratory failure (Raghu et al. 2011). In the past, IPF was commonly considered an inflammatory-driven disease, nowadays it is rather identified as a primarily fibrotic process initially triggered by an epithelial chronic injury that causes an altered and exaggerated activation of fibroblasts. Although the pathogenesis of IPF is still unclear, genetic and epigenetic factors along with recognized risk factors such as smoking, are considered crucially important for the onset of the fibrotic process (Puglisi et al. 2016). These factors, together with repeated damage of the alveolar epithelium and an altered repairing process, are responsible for an abnormal activation, differentiation, and proliferation of mesenchymal cells. The origin of mesenchymal cells is variable, deriving from alveolar epithelium, resident fibroblasts, and circulating fibrocytes. Many cytokines such as TGF-β and PDGF actively contribute to the proliferation of fibroblasts and the differentiation of these cells into myofibroblasts (Sgalla et al. 2018; Spagnolo et al. 2015). Considering the large variety of pathogenic and clinical similarities, IPF has been, at least in some aspects, compared to cancer (Vancheri 2013).

So far only two drugs, pirfenidone, and nintedanib, have been approved for the treatment of IPF. Both drugs slow disease progression, increase survival but are not able to stop or reverse the relentless course of the disease (Fernández Fabrellas et al. 2018). For this reason, research is strongly focused on the identification of new targets amenable to therapeutic intervention.

4.2 Are the Expression and Functional Profile of PI3K Relevant to the Pathogenesis of IPF?

In the last few years, several studies have revealed an involvement of the PI3K pathway in the pathogenesis of IPF. In vitro studies have demonstrated, for the first time, the central role of this signaling pathway in regulating the proliferation and differentiation of human lung fibroblasts into myofibroblasts induced by TGFβ

(Conte et al. 2011). It was also described that PTEN negatively regulates myofibroblast differentiation both in vitro and in vivo (White et al. 2006; Xia et al. 2008). This was confirmed by Le Cras et al. who showed that treatment with the pan-PI3K inhibitor PX866**,** prevents the progression of lung fibrosis induced by TGFα in vivo (Cras et al. 2010). Specifically, the involvement of the PI3Kγ isoform in lung fibrogenesis was evaluated demonstrating that mice lacking PI3Kγ are protected against bleomycin-induced pulmonary injury and displayed higher survival rates than wild-type mice. AS605240, a specific inhibitor of PI3Kγ, also decreases the production of collagen in response to CXCL1 and CCL2 in lung fibroblasts of mice instilled with bleomycin (Russo et al. 2011). Furthermore, overexpression of the PI3Kγ isoform has been observed in IPF lung homogenates and in fibrotic human lung fibroblasts. Moreover, gene silencing and pharmacological inhibition of PI3Kγ with AS252424 significantly inhibited cell proliferation and α-SMA expression in IPF fibroblasts (Conte et al. 2013). Recently, omipalisib/GSK2126458**,** a selective inhibitor of class I PI3Ks and mTOR, has been repositioned from oncology to IPF for its anti-fibrotic effects. Biochemical activity studies revealed the specificity of this compound for the PI3Kα isoform showing as well some degree of activity against isoforms β, γ, and δ (Knight et al. 2010). Indeed, omipalisib decreased fibroblast proliferation and TGFβ-induced collagen synthesis in primary human lung fibroblast cultures. Moreover, omipalisib reduced AKT phosphorylation in IPF BAL cells and *ex vivo* IPF lung tissue (Mercer et al. 2016). Omipalisib has been also investigated in a phase 1 clinical trial (NCT01725139), with 17 IPF patients enrolled, where it displayed a good safety profile (Lukey et al. 2019).

4.3 Are PI3K Inhibitors Safe and Clinically Relevant in IPF?

A critical aspect of PI3K inhibitors is the induction of serious side effects, mainly hyperglycemia and gastrointestinal toxicity, which often result in treatment discontinuation. A very interesting approach with PI3K inhibitors for the treatment of IPF was made by Hettiarachchi et al. (2020). To increase efficacy, possibly diminishing toxicity associated to PI3K inhibitors, they thought to directly target myofibroblasts. Myofibroblasts, mainly present in fibrotic tissues, at the invasive front of cancer and during wound healing, express on their membrane a protein named fibroblast activation protein (FAP) that is virtually a specific marker for these cells (Acharya et al. 2006). Any molecule or drug associated to a ligand for FAP can be the ideal way to selectively target myofibroblasts. These authors, with the aim of inhibiting the activity of myofibroblasts in a selective manner, investigated the effect of omipalisid bound to a FAP ligand (FAPL) in the mouse model of bleomycin-induced fibrosis. With all the limitations of this model that resemble only in part to human IPF, the PI3K inhibitor plus the FAP ligand complex significantly reduced collagen production and the development of lung fibrosis (Hettiarachchi et al. 2020).

Campa et al. (2018) proposed an alternative strategy to reduce toxicity of PI3K inhibitors and maximize their therapeutic efficacy using local and not systemic

administration. CL27c, a prodrug pan-PI3K inhibitor, was chemically designed for local therapy and administered by inhalation. Its efficacy was investigated in a murine model of pulmonary fibrosis and asthma. CL27c inhaled, as an aerosol, once activated inside the cytoplasm of lung cells in its active form CL27e, inhibits PI3K activity only locally, minimizing the toxic effects of systemic exposure and possibly increasing efficacy. Indeed, CL27c was able to reduce lung damage and prevent animal mortality, even when administered in a therapeutic setting. Recently, 2-amino-4-methyquinazoline derivates (5d, 5e, and 5 g), a new class of PI3K inhibitors, have been synthesized and tested for their anti-proliferative activities in mouse MLg2908 lung fibroblasts, through the inhibition of the main PI3K downstream signaling pathway such as AKT, p70S6K, and S6RP. In an in vivo fibrotic model, oral administration of 5d suppressed the α-SMA and hydroxyproline increase in lung homogenates and improved the deterioration of lung function induced by bleomycin (Lin et al. 2019).

The PI3K/AKT/mTOR axis is also involved in the mechanism of autophagy. Autophagy is a physiological process that allows the elimination of misfolded proteins or damaged cell components by lysosomal vesicles that plays an important role in tissue development, defense against pathogens, and in counteracting cellular aging. An autophagy deficit has been linked to the development of several diseases, including IPF (Todde et al. 2009). In the course of IPF there are several factors that contribute to increased autophagy, including oxidative stress (Kliment and Oury 2010). TGFβ is one of the inducers of oxidative stress in IPF by increasing reactive oxygen species production and reducing the production of antioxidant enzymes, causing an imbalance in favor of ROS formation (Liu and Desai 2015). TGFβ inhibits autophagy in fibroblasts via the activation of the PI3K/AKT/mTOR axis, for that rapamycin administration results in decreased autophagy and increased fibrosis in bleomycin-induced models (Patel et al. 2012). Reduced autophagy results in decreased collagen degradation and proliferation of fibroblasts together with differentiation into myofibroblasts, cells with a pivotal role in the genesis of IPF (Zhao et al. 2020).

Based on these premises, the PI3K/AKT/mTOR axis could represent a promising potential therapeutic target for IPF. Lukey et al. (2019) investigated the role of omipalisib, in a randomized, placebo-controlled, double-blind study in patients with IPF. The study was exclusively addressed to test safety and pharmacokinetics. Omipalisib, given twice daily to the 17 patients enrolled in the study, exerted a dose-dependent inhibition of the PI3K/mTOR pathway both in blood and BAL cells. The safety profile was good, limited to mild diarrhea in four patients. Although there are no specific data on efficacy, it is interesting to note that 18F-2-fluoro-2-deoxy-D-glucose(FDG)-positron emission tomography/computed tomography scans showed an exposure-dependent reduction in 18F-FDG uptake in fibrotic areas of the lungs. The results of this study, although limited to the evaluation of safety and pharmacokinetics of omipalisib should encourage the launching of other clinical studies in this field. Unfortunately, to date, only one randomized controlled trial is ongoing in IPF. HEC68498, a potent, high selective inhibitor of class I PI3K and mTOR is now

under study in a phase I study. It is a double-blind, placebo-controlled trial, but no data are currently available (NCT03502902) (Clinical Trials gov 2021a).

4.4 Closing Remarks

Similar to bronchial asthma or COPD, the PI3K pathway is fully involved in all known pathogenic mechanisms underlying IPF. Many in vitro studies and in vivo animal models of lung fibrosis, clearly demonstrate that the inhibition of PI3K is related to the reduction of fibroblast activation, proliferation, and differentiation. In addition, the release of pro-fibrotic cytokines such as TGFβ or the production of collagen is downregulated. Despite this experimental evidence and a reasonably good safety profile, very few clinical trials have been designed for the evaluation of the clinical efficacy of PI3K inhibitors in IPF. Unfortunately, current therapies may prolong survival, but IPF remains a lethal disease. It is highly desirable that in near future more trials will be specifically designed to investigate the efficacy of PI3K inhibitors in IPF.

5 PI3K and Lung Cancer

5.1 Background

Lung cancer is the second more common cancer after breast cancer and represents the first cause of cancer mortality (Sung et al. 2021). Its classification was recently modified according to new knowledge regarding its molecular profile. It is divided into Small Cell Lung Carcinoma (SCLC), accounting for about 15% of all lung cancers, and Non-Small Cell Lung Carcinoma (NSCLC), including Adenocarcinoma, Squamous Cell Carcinoma, and Large Cell Carcinoma (Inamura 2017). Despite the efforts for an early diagnosis and the new therapeutic interventions, lung cancer remains frequently diagnosed in advanced stages, with a 5-year survival rate ranging to 20–25% (Leiro-Fernández et al. 2014). There is an urgent need for more effective treatments that could improve the current disappointing survival rates. In this view, the PI3K pathway is of great interest, due to its role in cell activation and replication.

5.2 Are PI3K Inhibitors Safe and Clinically Relevant in Lung Cancer?

Chemotherapy remains one of the main treatment strategies for lung cancer. In the last few years, the pharmacological treatment is switching from conventional

chemotherapy to a personalized approach, mainly based on markers of cell activity displayed by cancer cells or against molecules belonging to pivotal cancer pathways. In this view, the PI3K/AKT/mTOR pathway is one of the most studied. A great number of mutations or imbalances have been found in molecules involved in this pathway in lung cancer cells, due to either an inhibition of down-regulatory systems or to a chronic activation of the pathway. The use of compounds capable of inhibiting the PI3K pathway, alone or in combination with other treatments, can also provide a way to overcome chemotherapy resistance (Brown and Toker 2015).

Sonolisib (PX-866) is a pan-inhibitor of PI3K, analog of wortmannin, that displays significant antitumor activity in xenograft models and a good safety profile in advanced tumors. The phase I/II study in association with docetaxel in NSCLC is completed but results are still pending (Hong et al. 2012; Clinical Trials gov 2021b). Another pan-inhibitor of PI3K is buparlisib (BKM-120), which also shows some activity on the inhibition of tubulin (Zhao et al. 2017; Wu et al. 2016; Xing et al. 2021). The dose administered was well tolerated at 100 mg/day and tested in few clinical trials in NSCLC (Wu et al. 2016; Xing et al. 2021; Trials and gov 2021; Clinical Trials gov 2021c, a, d, e, f; McGowan et al. 2019), alone or in combination with other molecules. Finally, in this family of PI3K pan-inhibitors, pilaralisib (SAR245408, XL147) resulted safe but not sufficiently effective (Wheler et al. 2017). ASN003 is instead a dual, PI3Kα and δ isoform inhibitor with a potent inhibitory activity on BRAF, a serine/threonine kinase mutated in about 4% of NSCLC (Bustamante and Otterson 2019). A phase I study was completed, but no other clinical trials are currently available on this compound (Bustamante and Otterson 2019; Clinical Trials gov 2022b). Similar action, but limited to the inhibition of PI3Kα and δ is played by pictilisib, which showed a good safety profile in an IB study in association with the standard of care for NSCLC. Progressing to phase II, this study is currently ongoing (Soria et al. 2017; Clinical Trials gov 2021g). Another PI3K inhibitor is taselisib (GDC-0032), specific for the α, γ, and δ isoforms. The molecule was tested in several solid cancers, but when used alone in NSCLC did not sufficiently improve the survival rate (Langer et al. 2019). Eganelisib (IPI-549) is a selective inhibitor of PI3Kγ, an isoform mostly expressed in immune cells. This isoform plays a critical role in cancer microenvironment, increasing the activity of double-negative T cells, myeloid-derived suppressor cells, and tumor-associated macrophages with potent immunosuppressant effects, aimed to escape the anticancer surveillance of the immune system (Hillhouse et al. 2013; Zhang et al. 2019). This indirect mechanism was exploited in the treatment of melanoma and head/neck tumors with encouraging results (Cohen, et al. 2020; Postow, et al. 2020). A phase I clinical trial on Eganelisib plus nivolumab, enrolling NSCLC patients is currently ongoing (Clinical Trials gov 2021h). Two other compounds are currently studied with a selective inhibition for PI3Kα, i.e., alpelisib (BYL719) and serabelisib (TAK-117). Alpelisib was mainly tested to evaluate tolerability and preliminary efficacy in patients with advanced solid cancers including some patients with lung cancer. Hyperglycemia, cutaneous rash, loss of appetite and diarrhea were the most common, although manageable adverse event. Tumor shrinkage was described in some patients, but the overall number of participants and specifically of lung cancer patients was very low. The results of a

phase II study that included only two patients are still pending (Ando et al. 2019; Clinical Trials gov 2021i). Serabelisib (TAK-117) is being tested as part of an open phase II clinical trial in combination with canaglifozin, not yet recruiting to date (Juric et al. 2017; Clinical Trials gov 2021j).

BGB-10188 is a PI3Kδ-specific inhibitor (Yang et al. 2020). The phase I/II study, aimed to determine the maximum tolerated dose, included NSCLC patients, evaluating the compound alone or in combination with tislelizumab (Clinical Trials gov 2021k). Another PI3Kδ inhibitor with an excellent in vivo safety profile is Parsaclisib (INCB50465), for which a phase I clinical trial in combination with pembrolizumab was designed. The trial that involves lung cancer patients is still not currently recruiting (Yue et al. 2019; Clinical Trials gov 2021l). Finally, idelalisib, already approved for the treatment of lymphoma, is going to be tested in a phase I/II study in combination with pembrolizumab (Raedler 2015; Clinical Trials gov 2021m).

AZD8186 is considered a PI3Kβ inhibitor, although a weaker inhibition of PI3Kδ has been also described (Barlaam et al. 2015). In vitro studies on PTEN null tumors, proved efficacy of AZD8186 in the downregulation of cholesterol biosynthesis genes and upregulation of markers associated with metabolic stress (Lynch et al. 2017), but its action, when used alone in vivo, resulted limited (Owusu-Brackett et al. 2020). In this view, a phase I clinical trial involving NSCLC patients was carried out (Clinical Trials gov 2021n). In this trial, AZD8186 was used alone and in combination. The study result was completed, but the results are not available yet.

Some molecules have also shown the ability to inhibit the PI3K/AKT/mTOR pathway at multiple levels. Perifosine inhibits both PI3K and AKT, showing an interesting in vitro activity against lung cancer, colorectal cancer, and multiple myeloma (Richardson et al. 2012). It was tested in a phase II clinical trial completed in 2018, but the results of the study are not yet available (Clinical Trials gov 2021o). Gedatolisib (PF-05212384) is a potent pan-class I PI3K and mTOR inhibitor, with a manageable safety profile and antitumor activity in advanced stage NSCLC patients (Mallon et al. 2011). The study encourages two phase I clinical trials including lung cancer patients, alone or associating the compound with docetaxel, cisplatin, or dacomitinib, showing good safety profile and preliminary antitumor activity (Shapiro et al. 2015; Wainberg et al. 2016). However, a recent phase II clinical trial in SCLC proved no benefit for patients treated with this molecule (Udagawa et al. 2020). A similar compound named Voxtalisib (SARD245409/XL765) was tested in phase I clinical trials on advanced solid cancer patients including lung cancer, alone (Papadopoulos et al. 2014) or in combination with pimasertib (Schram et al. 2018). In the first study, the safety was acceptable, however, in the second study, the patients proved poor tolerability and limited antitumor activity.

5.3 Closing Remarks

The PI3K/AKT/mTOR pathway plays a pivotal role in the modulation of cellular proliferation, and therefore in cancer development. Several cancers, including lung cancer, are characterized by an abnormal regulation of this pathway. This can be due to a constitutive reduced efficacy of those mechanisms of control involved in the downregulation of the pathway (e.g., PTEN null mutation) or to a constitutive activation of PI3K/AKT. Exploiting PI3K/AKT/mTOR in lung cancer treatment seems a promising opportunity. However, to date none of the molecules tested progressed toward a phase III clinical trial. It must be said that some clinical trials raised safety concerns and above all registered limited results regarding clinical efficacy. Even so, there is still great interest for this therapeutic approach in lung cancer, proved by the elevated number of clinical trials currently ongoing. Hopefully, the results of these studies will add new and sufficient information for the development of new effective drugs for lung cancer. There is already a signal going into this direction. Some evidence in the treatment of cancer patients suggests the possibility that PI3K inhibitors may enhance the response to conventional treatment (Wright et al. 2021), however, the definitive utility of these molecules in combination to standard treatment needs to be further addressed by additional studies.

6 PI3K and ARDS

6.1 Background

Acute respiratory distress syndrome (ARDS) is an inflammatory disease caused by an uncontrolled systemic and/or local pulmonary inflammatory response due to the effects of risk factors that may involve the lung either directly (toxic gas inhalation, aspiration of gastric content, severe lung infection, etc.) or indirectly (systemic infection, severe trauma, drug overdose, multiple blood transfusion, etc.). These pathological conditions may induce a systemic and local inflammatory response causing inflammatory alveolar damage and pulmonary, non-cardiogenic, edema. From the clinical point of view, ARDS is characterized by acute and progressively increasing dyspnea with severe hypoxemia leading to death in about 50% of cases (Wheeler and Bernard 2007; Thompson et al. 2017). The pathogenic basis of ARDS includes a variety of complex mechanisms that can be summarized in an excessive activation of immune cells, dysregulated inflammation, and increased lung endothelial and epithelial permeability that cause the disruption of the lung microvascular barrier (Huppert et al. 2019; Sapru et al. 2015).

6.2 Are the Expression and Functional Profile of PI3K Relevant to the Pathogenesis of ARDS?

Studies on the role of PI3K/AKT/mTOR pathway on ARDS were mainly focused on the role of the regulatory protein PTEN. Murine, epithelium-specific PTEN-deleted models showed severe hypoxia, increased alveolar permeability, alveolar flooding, and lung fibrosis. These models also showed a reduction of the production of claudin-4, a key factor for the integrity of tight junctions, and increased levels of activated AKT and MMP2. The result is an impairment of the integrity of the alveolus-capillary membrane barrier (Yanagi et al. 2015). These results have also suggested that PI3K could be a potential target for ventilator-induced ARDS. Over-ventilated, isolated lungs from PI3K$\gamma^{-/-}$ mice, showed decreased phosphorylation of AKT as well as histological changes typical of lung injury (Lionetti et al. 2006). The involvement of the PI3Kγ isoform was also emphasized in an endotoxin-induced model of acute lung injury. Specifically, the severity of endotoxemia-induced ARDS and lung neutrophil accumulation were significantly reduced in mice lacking the PI3K p110γ catalytic subunit compared with wild-type mice (Yum et al. 2001). The close association of PI3Kδ and injury-induced lung damage has been also extensively studied, although its role has been only recently recognized in ARDS. In this regard, the "Activated PI3Kδ syndrome" (APDS) has been described, a dominant gain-of-function mutation of the *PIK3CD* gene. The mutated gene was identified in 17 patients, with recurrent respiratory infections, progressive airway damage, lymphopenia, impaired vaccine responses, increased circulating transitional B cells, increased serum levels of IgM and decreased levels of IgG2. Interestingly, these patients also showed high levels of phosphatidylinositol-3-phosphate (PIP3) and phosphorylated AKT. IC87114 and GS-1101, two different selective PI3Kδ blockers, inhibited AKT activity in $CD4^+$ and $CD8^+$ T lymphocytes both on wild type and mutated PI3Kδ in vitro (Angulo et al. 2013). The involvement of PI3Kδ in the modulation of airway neutrophil recruitment and bronchial epithelial functional alterations has been also investigated in a murine model of acute lung injury (ALI). In the LPS-induced model of lung injury, the pharmacological inhibition of PI3Kδ with either IC87114 or AMG319 mitigated LPS-induced edema, lung injury, and neutrophilic inflammation, and increased TNF and IL-6 levels in BAL were also diminished (Yao et al. 2021).

6.3 Are PI3K Inhibitors Safe and Clinically Relevant in ARDS?

Evidence in the treatment of Acute Lung Injury (ALI)/ARDS through the inhibition of the PI3K pathway is still limited and despite some pathophysiological evidence, to the best of our knowledge, no clinical trials aimed to evaluate the inhibition of this pathway in ALI/ARDS are ongoing.

However, more recently PI3Kδ was suggested as a possible target for COVID-19-related ARDS (Zhang et al. 2020). Idelalisib, currently used in hematologic malignancies and studied in allergic diseases, was suggested as a potential pharmacological therapy for COVID-19, alone or in combination with ebastine for their capacity to decrease inflammatory processes (Palma et al. 2020). Macrolides are also widely used in the treatment of COVID-19 patients, being in some instances efficacious mainly by preventing disease progression (Batiha et al. 2021). This action could be mediated by the effect of macrolides at inhibiting PI3K. Their use could be reasonable due to the well-known safety profile and its activity in preventing bacterial super-infection (Bacharier et al. 2015).

Currently, several ongoing clinical trials are testing the inhibition of PI3K/AKT/mTOR in the treatment of COVID-19. Drugs currently studied are duvelisib, a dual inhibitor of PI3Kγ/δ (Clinical Trials gov 2021p), and sirolimus, an mTOR inhibitor (Clinical Trials gov 2021q, r, s, t).

6.4 Closing Remarks

A considerable amount of data suggests that PI3K signaling and its downstream mediators are strongly activated in lung injury and ARDS. However, very few clinical data are currently supporting the use of PI3K inhibitors in a clinical setting. Certainly, the disappointing current survival of patients with ARDS will spur the design of new trials investigating different therapeutic approaches and among these, the inhibition of the PI3K axis seems promising.

7 General Conclusions

Many studies have clearly demonstrated that PI3K is fully involved in most of the steps underlying the pathogenesis of respiratory diseases such as bronchial asthma, COPD, IPF, lung cancer, and ARDS. This is not surprising considering the important regulatory role exerted by PI3K in a wide array of cellular activities such as growth, proliferation, differentiation, cytoskeletal organization, migration/adhesion, survival/apoptosis, and angiogenesis. The use of non-selective and even more isoform-selective PI3K inhibitors has allowed a better understanding of the specific roles played by each isoform in various lung disorders. Several in vitro and in vivo animal models of asthma, COPD, IPF, lung cancer, and ARDS have offered enough evidence to design clinical trials to evaluate the safety profile and efficacy of this therapeutic approach. Nevertheless, few clinical trials have been performed so far with the exception of lung cancer, where a discrete number of trials are currently ongoing. All studies had as primary outcome drug tolerability and only in a few cases, efficacy was assessed. In general, the safety profile was good and limited to manageable adverse events. This was particularly evident when inhibitors were

administered locally, by inhalation. In contrast, efficacy data, registered so far, are not brilliant and need further larger and well-powered studies. It is evident that new clinical trials designed for the evaluation of efficacy are not easy to perform. Many variables need to be considered, including the possibility that the positive effect of blocking PI3K in the clinical setting could not be enough to achieve a clinical result. As often happens in biology, redundant signaling pathways could intervene mitigating a potential positive effect. Most of the respiratory diseases described in this chapter are marked by low survival rates and all need a substantial improvement of the current medical treatments. The history of the treatment of respiratory disease with PI3K inhibitors is just at its beginning and certainly needs additional studies and further efforts from researchers and clinicians involved in this fascinating field.

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