Distinct roles of class IA PI3K isoforms in primary and immortalised macrophages

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Summary

The class IA isoforms of phosphoinositide 3-kinase (p110α, p110β and p110δ) often have non-redundant functions in a given cell type. However, for reasons that are unclear, the role of a specific PI3K isoform can vary between cell types. Here, we compare the relative contributions of PI3K isoforms in primary and immortalised macrophages. In primary macrophages stimulated with the tyrosine kinase ligand colony-stimulating factor 1 (CSF1), all class IA PI3K isoforms participate in the regulation of Rac1, whereas p110δ selectively controls the activities of Akt, RhoA and PTEN, in addition to controlling proliferation and chemotaxis. The prominent role of p110δ in these cells correlates with it being the main PI3K isoform that is recruited to the activated CSF1 receptor (CSF1R). In immortalised BAC1.2F5 macrophages, however, the CSF1R also engages p110α, which takes up a more prominent role in CSF1R signalling, in processes including Akt phosphorylation and regulation of DNA synthesis. Cell migration, however, remains dependent mainly on p110δ. In other immortalised macrophage cell lines, such as IC-21 and J774.2, p110α also becomes more prominently involved in CSF1-induced Akt phosphorylation, at the expense of p110δ. These data show that PI3K isoforms can be differentially regulated in distinct cellular contexts, with the dominant role of the p110δ isoform in Akt phosphorylation and proliferation being lost upon cell immortalisation. These findings suggest that p110δ-selective PI3K inhibitors may be more effective in inflammation than in cancer.
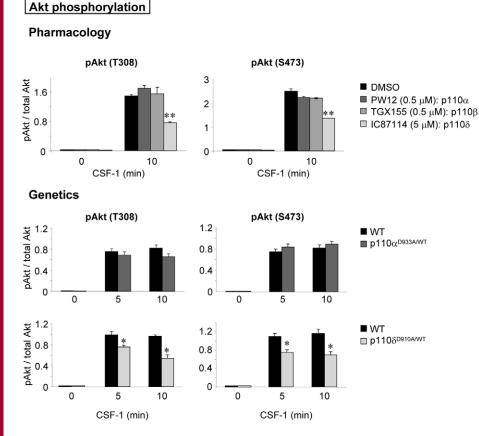
Introduction

Class IA phosphoinositide 3-kinases (PI3Ks) are heterodimers consisting of a catalytic subunit (p110α, p110β or p110δ) in complex with one of five regulatory subunits (collectively called the 'p85s'). The interaction of the Src homology 2 (SH2) domains of p85 with phosphotyrosine residues in receptors and adaptor molecules facilitates recruitment of the class IA PI3Ks to the membrane, where they generate lipid second messenger signals that control cell growth, proliferation, survival, intracellular traffic, cytoskeletal changes and cell migration (Vanhaesebroeck et al., 2001). Whereas p110α and p110β are ubiquitously expressed, p110δ expression is low in most cells (Sawyer et al., 2003) but highly enriched in leukocytes (Chantry et al., 1997; Vanhaesebroeck et al., 1997) and to a lesser extent in neurons (Eickholt et al., 2007). Some cancer cell lines, including some of breast and melanoma origin, can also express high levels of p110δ (Arcaro et al., 2002; Boller et al., 2008; Chaussade et al., 2007; Sawyer et al., 2003).

We and others have presented evidence that the class IA PI3K isoforms often exert distinct biological roles downstream of specific receptors in various cell types. In early experiments, evidence for such non-redundancy at the cellular level was obtained by microinjection of PI3K-isoform-specific antibodies in cells (Bénistant et al., 2000; Hooshmand-Rad et al., 2000; Leverrier et al., 2003; Roche et al., 1994; Sawyer et al., 2003; Vanhaesebroeck

et al., 1999; Windmiller and Backer, 2003; Yip et al., 2004). More recently, non-redundancy of PI3Ks was also observed in cells derived from PI3K gene-targeted mice and by the use of isoform-selective small molecule inhibitors (Ali et al., 2004; Eickholt et al., 2007; Foukas et al., 2006; Knight et al., 2006; Okkenhaug et al., 2002). Overexpression of PI3Ks in avian fibroblasts also shows remarkable differences in signalling and biological output between different PI3K isoforms (Denley et al., 2007; Kang et al., 2006).

However, it is also clear that a given PI3K isoform can take up distinct roles in different cellular contexts, and thus it has not been possible to link a specific biological response (such as DNA synthesis) to a single PI3K isoform. One possible explanation for the distinct roles of PI3K isoforms in different cell types could be their relative expression levels. For example, p110α was found to be dispensable for stimulus-induced actin cytoskeletal changes in MDA-MB-231 breast cancer cells, which were instead controlled by p110δ (Sawyer et al., 2003). However, in rat MTLn3 adenocarcinoma cells, it is p110α that regulates cytoskeletal changes, possibly because these cells lack detectable expression of p110δ (Hill et al., 2000). Consistent with this hypothesis, in endothelial cells, which express low levels of p110δ, it is p110α that regulates cell migration (Graupera et al., 2008).

Another example where the relative expression levels of PI3K isoforms can contribute to the relative importance of biological

Fig. 1. Effect of pharmacological or genetic inactivation of class IA PI3K isoforms on CSF1-induced phosphorylation of Akt. Upper panel, WT BMMs were pre-treated for 1 hour with PW12 (0.5 µM), TGX155 (0.5 µM) or IC87114 (5 µM), followed by incubation with CSF1 (30 ng/ml) for 10 minutes and analysis of phosphorylation of Akt (on T308 and S473) by western blotting of total cell lysates (80 µg/lane). Graph represents the mean ± s.e.m. of a representative experiment performed in triplicate (**$P<0.01$). Lower panel, BMMs with heterozygotic inactivation of p110α or p110δ were stimulated with 30 ng/ml CSF1 for the indicated time points, followed by analysis as described above. Graphs represent the mean ± s.e.m. of three separate experiments (*$P<0.05$).

functions is the involvement of p110 isoforms in insulin signalling. Indeed, whereas p110α appears to be the main PI3K isoform that controls insulin signalling in primary tissues and several cell lines (Foukas et al., 2006; Knight et al., 2006), this is not the case in the CHO-IR (Chinese Hamster Ovary cells that overexpress the insulin receptor), HepG2 hepatoma and J774.2 macrophage cell lines in which p110β and even p110δ take a more prominent role (Chaussade et al., 2007; Hooshmand-Rad et al., 2000), often correlating with their increased expression relative to p110α (Chaussade et al., 2007). However, expression levels of p110 isoforms cannot fully explain isoform-selective functions. For example, in primary tissues, p110β is often present in substantially higher absolute amounts than p110α (Geering et al., 2007), yet does not participate in early insulin signalling (Foukas et al., 2006).

Using an antibody microinjection approach, we previously presented evidence for non-redundancy of the class IA PI3K isoforms in immortalised BAC1.2F5 macrophages. In these cells, p110α solely controls DNA synthesis while playing no part in regulating actin cytoskeletal changes and chemotaxis, which are regulated instead by p110β and p110δ (Vanhaesebroeck et al., 1999). Subsequently, we have used isoform-selective small-molecule inhibitors for PI3K and cells from PI3K-gene-targeted mice to investigate the functions of PI3K isoforms in primary macrophages, and found that p110δ controls both DNA synthesis and migration (Papakonstanti et al., 2007). The latter observation was somewhat surprising given that p110δ appears not to be involved in regulating DNA synthesis in BAC1.2F5 cells (Vanhaesebroeck et al., 1999), but in line with the key role of p110δ in proliferation induced by, for example, c-kit ligand in primary mast cells (Ali et al., 2004; Ali et al., 2008) and antigen in lymphocytes (Bilancio et al., 2006;

Clayton et al., 2002; Jou et al., 2002; Okkenhaug et al., 2002). In the current study, we dissected and compared the roles of the class IA PI3K isoforms in signalling and biological activities in primary and immortalised macrophages. We found that some responses in a given cell type (such as stimulation of Rac1 in primary macrophages) can be shared by all PI3K isoforms, whereas other functions (such as regulation of Akt, RhoA and PTEN) are isoform-specific. The relative importance of a given PI3K isoform can also change. For example, p110α has a much more substantial role in the regulation of DNA synthesis in immortalised macrophages compared with primary macrophages. Differential recruitment of PI3K isoforms to signalling complexes and other possible mechanisms for non-redundancy are discussed.

Results

Inactivation of class IA PI3K isoforms in macrophages

To pharmacologically interfere with the activity of PI3K isoforms in wild-type (WT) bone marrow macrophages (BMMs), we used the small molecule inhibitors PW12, TGX155 and IC87114, which have

Table 1. In vitro IC$_{50}$ (µM) of compounds that inhibit class I PI3K isoforms

	p110α	p110β	p110δ	p110γ
PW12	0.015	0.83	0.73	0.97
TGX-155	7.2	0.030	0.38	14.1
IC87114	>100	38	0.033	0.7
LY294002	0.85	0.24	0.68	3.1

Assays were conducted side by side using 10 µM ATP and 100 µg/ml phosphatidylinositol as the substrate.
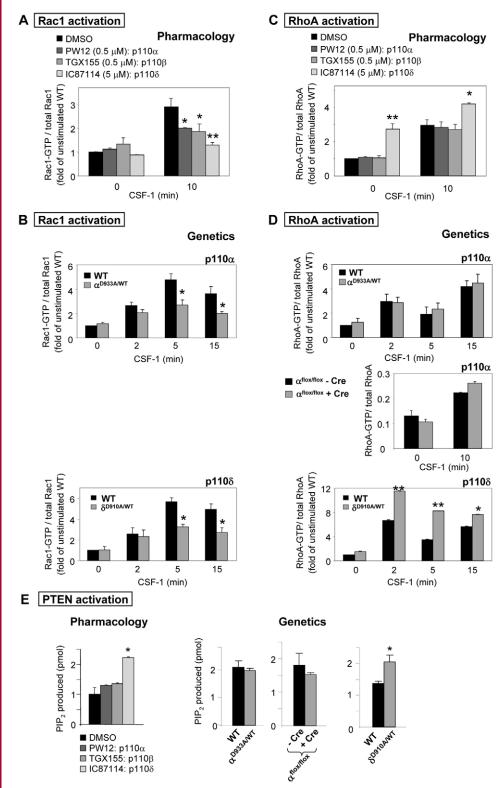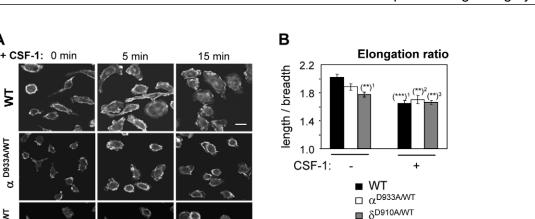
Fig. 2. Effect of pharmacological or genetic inactivation of class IA PI3K isoforms on the activation of small GTPases and PTEN. (A,B) Equal volumes of cell lysates of the indicated BMMs were subjected to pull-down assay with GTP-PBD, followed by detection of precipitated Rac1 by western blotting. Total cell lysates were resolved and immunoblotted for Rac1. Graphs represent the mean ± s.e.m. of three experiments. *$P<0.05$; **$P<0.01$ when compared with cells treated with vehicle only for each time point or with WT cells. (C,D) Equal volumes of cell lysates of the indicated BMMs were subjected to pull-down assay with GST-RBD, followed by western blot detection of precipitated RhoA. Total cell lysates were resolved on the same SDS-PAGE gel and immunoblotted for RhoA. Graphs for BMMs represent the mean ± s.e.m. of three experiments. *$P<0.05$; **$P<0.01$, compared with cells treated with vehicle only for each time point or with WT cells. (E) BMMs were pre-treated for 1 h with PW12 (0.5 μM), TGX155 (0.5 μM) or IC87114 (5 μM), followed by assay of PTEN lipid phosphatase activity as described (left panel). One representative experiment done in triplicate is shown (*$P<0.05$). Right panels show the effect of genetic inactivation of p110δ or p110α on PTEN lipid phosphatase activity. PTEN was immunoprecipitated from the respective BMM lysates followed by determination of its phosphatase activity towards synthetic PIP3 by ELISA (Echelon). One representative experiment done in triplicate is shown (*$P<0.05$).

selectivity for p110α, p110β and p110δ, respectively (Table 1). We also derived BMMs from mice with inactive germline alleles of p110α [p110α^{D933A} (Foukas et al., 2006)] or p110δ [p110δ^{D910A} (Okkenhaug et al., 2002)], using cells derived from WT mice as controls. Given that the function of p110α and p110δ is dose-dependent (Bilancio et al., 2006; Foukas et al., 2006), we also cultured cells from mice heterozygous for these alleles (further referred to as α$^{D933A/WT}$ and

δ$^{D910A/WT}$, respectively). We also conditionally inactivated p110α in macrophages, given that homozygous p110α$^{D933A/D933A}$ mice are embryonic lethal at mid-gestation (Foukas et al., 2006). This was done by tamoxifen treatment of fully differentiated macrophages derived from *Rosa26CreERT²*/p110α$^{flox/flox}$ mice (Graupera et al., 2008), resulting in acute activation of the Cre recombinase and ablation of p110α expression.

Fig. 3. Effect of genetic inactivation of p110α or p110δ on basal and CSF1-induced morphological changes in BMMs. (A) CSF1-starved or CSF1-stimulated cells were stained for F-actin. Scale bar: 30 μm. (B-D) Measurement of elongation ratio (ratio of the longest to the shortest cell axes, B), total area of adhesion (C) and ruffling (D). The results shown are mean ± s.e.m. of ≥180 cells from each population. ***$P<0.0001$; **$P<0.05$. Comparison labelled 1 was with unstimulated WT; 2 with unstimulated α[D933A/WT]; and 3 with unstimulated δ[D910A/WT] cells.

In primary macrophages, all class IA PI3K isoforms are involved in the regulation of Rac1 whereas p110δ selectively regulates Akt, RhoA and PTEN

Pharmacological inhibition of p110δ, but not of p110α or p110β, reduced the phosphorylation of Akt induced by CSF1 in primary BMMs (Fig. 1; supplementary material Fig. S1A). A similar selective role of p110δ was observed upon genetic inactivation of p110α or p110δ (Fig. 1; supplementary material Fig. S1B). The phosphorylation of Erk1/2 was not affected by either p110α or p110δ inactivation (supplementary material Fig. S1B).

We previously reported that p110δ positively regulates Rac1, and negatively regulates RhoA in primary macrophages (Papakonstanti et al., 2007). We now investigate whether p110α or p110β have similar roles in these cells. Pharmacological inactivation of each of the class IA PI3K isoforms led to a reduction in Rac1 activation in CSF1-stimulated macrophages (Fig. 2A; supplementary material Fig. S2A). Similar results were obtained upon genetic inactivation of p110α or p110δ (Fig. 2B; supplementary material Fig. S2B).

In line with our previous report (Papakonstanti et al., 2007), pharmacological inhibition of p110δ by IC87114 increased the cellular levels of Rho-GTP, both under basal and CSF1-stimulated conditions (Fig. 2C; supplementary material Fig. S2C). By contrast, pharmacological blockade of p110α or p110β did not affect RhoA activity (Fig. 2C; supplementary material Fig. S1C). Similar results were observed upon genetic inactivation of p110α or p110δ (Fig. 2D; supplementary material Fig. S2D,E).

We previously showed that PTEN is controlled by RhoA through a p110δ-dependent pathway (Papakonstanti et al., 2007). In line with the lack of an effect of p110α or p110β inhibition on RhoA, pharmacological blockade of these PI3K isoforms did not

affect PTEN activity, in contrast to inhibition of p110δ by IC87114, which led to PTEN activation (Fig. 2E, left panel). PTEN activity also remained unaffected upon genetic inactivation of p110α, in contrast to its constitutive increase in δ[D910A/WT] BMMs (Fig. 2E, right panel).

p110δ but not p110α controls chemotaxis of primary BMMs and immortalised BAC1.2F5 macrophages

Under CSF1-starved conditions, α[D933A/WT] and δ[D910A/WT] BMMs were more rounded compared with WT cells (Fig. 3A), in line with a reduced ratio of length over breadth (Fig. 3B). Under these basal conditions, the overall adhesive area of the cells was not affected upon inactivation of p110α or p110δ (Fig. 3C). Consistent with both p110α and p110δ controlling Rac1 activity (Fig. 2A,B), both isoforms were found to inhibit the acute morphological changes induced by CSF1, with reduced spreading (Fig. 3A,C) and membrane ruffling (Fig. 3D) in α[D933A/WT] and δ[D910A/WT] BMMs.

We next investigated the ability of BMMs to migrate up a linear concentration gradient of CSF1 in a Dunn chemotaxis chamber (Allen et al., 1998; Zicha et al., 1991). Time-lapse microscopy showed that α[D933A/WT] BMMs, in contrast to δ[D910A/WT] BMMs, were able to chemotax towards the source of CSF1 (Fig. 4A). Taken together, these observations (summarised in Fig. 4B) indicate that regulation of Rac1 is not crucial for the capacity of cells to perform chemotaxis, which is consistent with the normal chemotaxis in Rac1/2-null BMMs (Wheeler et al., 2006).
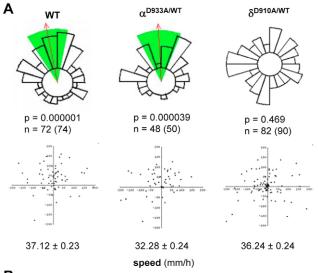
We previously investigated the role of PI3K isoforms using antibody-mediated neutralisation of PI3Ks in immortalised BAC1.2F5 macrophages (Boocock et al., 1989; Schwarzbaum et al., 1984), which have a bi-allelic deletion of the *Ink4a-Arf* locus (Quelle et al., 1995; Randle et al., 2001). Using these cells, we
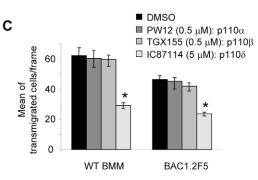
A

WT	$\alpha^{D933A/WT}$	$\delta^{D910A/WT}$
p = 0.000001	p = 0.000039	p = 0.469
n = 72 (74)	n = 48 (50)	n = 82 (90)

| 37.12 ± 0.23 | 32.28 ± 0.24 | 36.24 ± 0.24 |

speed (mm/h)

B

	$\alpha^{D933A/WT}$		$\delta^{D910A/WT}$	
	basal	+ CSF-1	basal	+ CSF-1
Rac1-GTP	no effect	↓	no effect	↓
RhoA-GTP	no effect	no effect	↑	↑
capacity to chemotax	yes		no	

C

- ■ DMSO
- ■ PW12 (0.5 μM): p110α
- □ TGX155 (0.5 μM): p110β
- □ IC87114 (5 μM): p110δ

Mean of transmigrated cells/frame

WT BMM   BAC1.2F5

	Impact of neutralisation of p110 isoforms on CSF-1-induced migration	
	primary BMM	BAC1.2F5
p110α	no effect	no effect
p110β	no effect	no effect
p110δ	↓	↓

Fig. 4. Effect of genetic inactivation of p110α or p110δ on chemotaxis of BMMs. (A) Chemotaxis of BMMs exposed to a gradient of CSF1 in Dunn chambers, monitored for 16 hours by time-lapse microscopy. The cell tracks from three separate experiments were merged into a single file for analysis. Circular histograms (upper panels) show the proportion of cells migrating into each of 20 segments of the angular trajectory plot (measured when each cell migrated past a horizon of 30 μm from its starting point with the source of CSF1 at the top). Arrows indicate significant mean directionality of the cell population. The green shaded areas mark the 95% confidence intervals of statistical significance. Similar chemotaxis plots were obtained for horizon limits of 50, 80, 100 μm, although the cell numbers varied (not shown). Vector plots (lower panel) show the end point of the cells with the starting point of each cell at the intersection between *x* and *y* axes and with the source of CSF1 at the top of each plot. (B) Summary of the effect of genetic inactivation of p110α or p110δ on the activation of Rac1 and RhoA, and on chemotaxis. (C) Effect of pharmacological inactivation of PI3K isoforms on chemotaxis in BMMs and BAC1.2F5 cells. Cells on the lower surface of the top chamber were stained and counted from at least six randomly chosen frames. *P<0.05 compared with DMSO-treated cells.

CSF1-induced DNA synthesis and Akt phosphorylation is controlled by distinct PI3K isoforms in primary and immortalised macrophages

Using antibody-mediated neutralisation of PI3K isoforms in BAC1.2F5 cells, we found that p110δ does not have a role in DNA synthesis in response to a saturating dose (33 ng/ml) of CSF1 (Vanhaesebroeck et al., 1999). In line with these data, inactivation of p110δ by IC87114 only had a very modest effect on CSF1-induced proliferation of these cells, especially at saturating CSF1 concentrations (Fig. 5A, left panel). In these cells, p110α appears to play a more prominent role in CSF1-induced DNA synthesis, as shown by antibody microinjection (Vanhaesebroeck et al., 1999) and pharmacological inhibition of p110α with PW12 (Fig. 5B).

Remarkably, PW12 had no effect on proliferation of primary macrophages (Fig. 5B), in line with the lack of an effect of genetic inactivation of p110α in these cells (Fig. 5C) (compare ±Cre) (supplementary material Fig. S3). In primary BMMs, p110δ has a more prominent role in the control of DNA synthesis, as shown by results from pharmacological (Fig. 5A,B,C) and genetic (supplementary material Fig. S3) approaches.

Inhibition of p110β by TGX155 did not affect DNA synthesis in either cell type (Fig. 5B), consistent with the observation that there is no change in DNA synthesis of BAC1.2F5 cells upon microinjection of antibodies to p110β (Vanhaesebroeck et al., 1999) or genetic inactivation of p110β in BMMs (Guillermet-Guibert et al., 2008) (and our unpublished results). Taken together, these data show that class IA isoforms of PI3K differentially contribute to CSF1-induced DNA synthesis in macrophages (summarised in Fig. 5D), with distinct isoforms having different roles in primary and immortalised macrophages.

We next sought to determine the roles of class IA PI3K isoforms in additional immortalised macrophage cell lines, including IC-21 (Mauel and Defendi, 1971), J774.2 (Kaplan and Morland, 1978; Morland and Kaplan, 1978) and RAW 264.7 (Raschke et al., 1978). Unlike BAC1.2F5, these cell lines do not depend on CSF1 for their proliferation (data not shown) but show a clear CSF1-induced phosphorylation of Akt (Fig. 6) and MAPK (data not shown). Inhibition of p110α strongly decreased Akt phosphorylation in BAC1.2F5, with no effect of inhibition of p110β or p110δ (Fig. 6;

documented that p110δ has a key role in regulating migration in response to a saturating dose (33 ng/ml) of CSF1 (Vanhaesebroeck et al., 1999). Using small-molecule inhibitors against PI3K isoforms rather than neutralising antibodies for analysis in Transwell chambers, we found that chemotaxis of BAC1.2F5 cells depends on p110δ (Fig. 4C), in line with previous data obtained by p110δ antibody micro-injection (Vanhaesebroeck et al., 1999), with no detectable role for p110α and p110β (Fig. 4C).
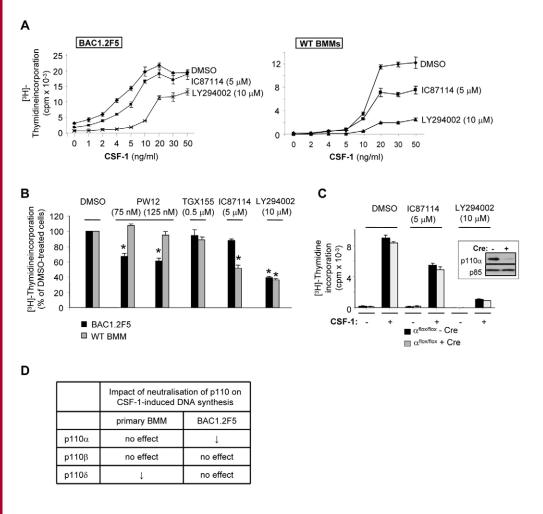
Fig. 5. Effect of pharmacological or genetic inactivation of class IA PI3K isoforms on DNA synthesis in BAC1.2F5 cells and BMMs. (A) Effect of the p110δ inhibitor IC87114 or the pan-PI3K inhibitor LY294002 on DNA synthesis induced in BAC1.2F5 and WT BMM cells by increasing doses of CSF1. (B) Effect of pharmacological inactivation of p110α, p110β or p110δ on DNA synthesis in BMMs and BAC1.2F5 cells induced by CSF1 (30 ng/ml). [³H]Thymidine incorporation of inhibitor-treated cells was expressed relative to that of cells treated with vehicle (DMSO) only (set as 100%). *$P<0.05$, compared with DMSO-treated cells. (C) Effect of homozygous deletion of p110α on DNA synthesis in BMMs. The inset shows the expression level of p110α in Cre⁻ and Cre⁺ BMMs in a representative experiment. (D) Summary of impact of p110 isoform neutralisation on CSF1-induced DNA synthesis in macrophages.

supplementary material Fig. S4). In the other immortalised cell lines, the role of p110δ in Akt phosphorylation was also found to be minimal to absent, with p110α taking up the most prominent role in IC-21 and J774.2 cells. Calculating the percentage inhibition observed upon blockade of each PI3K isoform in isolation suggests that full inhibition of Akt phosphorylation can be achieved by blockade of all class IA PI3K isoforms. Surprisingly, in RAW 264.7 cells, inhibition of individual PI3K isoforms did not significantly impact Akt phosphorylation (Fig. 6; supplementary material Fig. S4). The reason for this is currently unclear.

Taken together, these data show a general but variable difference between BMM and immortalised macrophage cell lines in their utilisation of p110 isoforms.

Differential recruitment of class IA PI3K isoforms to the activated CSF1R in primary and immortalised macrophages

The above results indicate that, for responses such as DNA synthesis and Akt phosphorylation, primary macrophages respond differently to immortalised BAC1.2F5 cells to the inactivation of PI3K isoforms, despite both cell types expressing similar levels of these proteins (Fig. 7A). *PIK3CA*, encoding p110α, is often mutated in cancer (Samuels et al., 2004). This was not the case in BAC1.2F5 cells, which carry a wild-type PIKCA, as verified by *PIK3CA* cDNA sequencing (data not shown). We therefore assessed recruitment of the distinct p110 isoforms to the activated CSF1R by immunoprecipitation of each p110 isoform from CSF1-stimulated macrophages, followed by western blotting for CSF1R or p85.

In primary BMMs, all immunoprecipitations recovered similar amounts of p85; however, p110δ immunoprecipitates clearly contained far more CSF1R than did those of p110α or p110β (Fig. 7B), indicating selective recruitment of p110δ over the other p110 isoforms to the activated CSF1R in these cells. This is in marked contrast to BAC1.2F5 cells, in which all PI3K isoforms, including p110α, became effectively recruited to the CSF1R (Fig. 7B, upper panel). In some experiments in BAC1.2F5 cells, p110α was bound to the CSF1R without prior CSF1 stimulation (Fig. 7B, lower panel). This may be due to incomplete CSF1 starvation, but demonstrates that under these conditions, p110α associates more prominently than p110β and p110δ with the CSF1R (Fig. 7B, lower panel).

Discussion
In this study, we first assessed the roles of the class IA PI3Ks in regulation of Rac1 and RhoA in primary macrophages. We previously documented that RhoA activity is increased upon inactivation of p110δ in diverse p110δ-expressing cells, such as primary and transformed macrophages, breast cancer cells (Papakonstanti et al., 2007) and neurons (Eickholt et al., 2007). We now show that this negative regulation of RhoA, at least in primary macrophages, is a p110δ isoform-specific function, as is the regulation of Akt and PTEN. This is in contrast to Rac1, which is controlled by all class IA PI3K isoforms (Fig. 2B,C). The reason for this differential PI3K isoform linkage of Rac1 compared with Akt/RhoA/PTEN is currently unclear. It is possible that Rac1 is more sensitive to the levels of PIP3, and is affected by very subtle
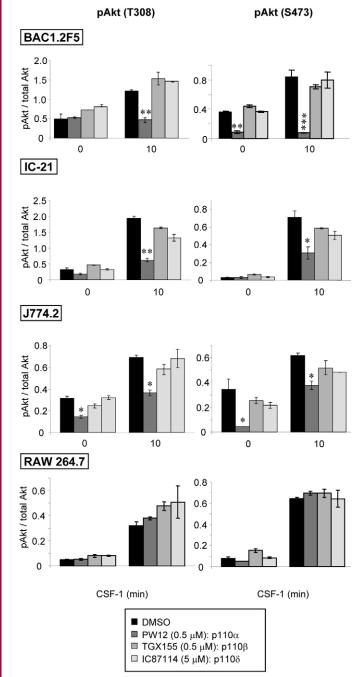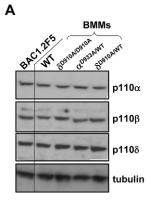
Fig. 6. Effect of pharmacological inactivation of class IA PI3K isoforms on CSF1-induced phosphorylation of Akt in macrophage cell lines. Cells were pre-treated for 1 hour with PW12 (0.5 μM), TGX155 (0.5 μM) or IC87114 (5 μM), followed by incubation with CSF1 (30 ng/ml) for 10 minutes and analysis of phosphorylation of Akt (on T308 and S473) by western blotting of total cell lysates (80 μg/lane). Graph represents the mean ± s.e.m. of two experiments performed in duplicate (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

alterations in any PI3K isoform. Rac has been extensively studied as a downstream target of PI3K, especially in the regulation of chemotaxis (Barber and Welch, 2006; Hawkins et al., 1995; Hooshmand-Rad et al., 1997; Kunisaki et al., 2006; Park et al., 2004; Wang, 2002; Xu et al., 2003). The observation that all class IA PI3K isoforms regulate Rac1 equally well, but that chemotaxis is

only affected upon inactivation of p110δ, suggests that Rac is not essential for chemotaxis, substantiating our previous observations in Rac1/2-null BMMs (Wells et al., 2004; Wheeler et al., 2006). It is tempting to speculate that RhoA, which is selectively controlled by p110δ, is a more important determinant of chemotaxis.

We further compared the function and receptor coupling of the class IA PI3K isoforms in primary or immortalised macrophages. In primary macrophages, as in primary mast cells and lymphocytes (Ali et al., 2004; Ali et al., 2008; Okkenhaug et al., 2002; Bilancio et al., 2006), p110δ is functionally dominant over the other class IA PI3K isoforms in all biological responses investigated thus far [our unpublished results; Klaus Okkenhaug (The Babraham Institute, Cambridge, UK), personal communication]. We previously documented that the role of p110δ becomes less important in a B cell lymphoma cell line compared with primary untransformed B cells (Bilancio et al., 2006). Similar observations are made in the current study. Indeed, although the isoform-selective role of p110δ in chemotaxis appears to be retained upon cell immortalisation (such as in BAC1.2F5 cells) (Fig. 4C), the contribution of p110δ to DNA synthesis and Akt phosphorylation becomes less important, with p110α taking on a more prominent role (Fig. 5D; Fig. 6). In other immortalised macrophage cell lines, Akt phosphorylation also becomes less dependent on p110δ, with a more prominent role for p110α and a modest role, if any role, for p110β (Fig. 6). Interestingly, p110β does not participate in CSF1-induced proliferation of either primary macrophages or BAC1.2F5 cells (Fig. 5B), the latter confirming our previous data obtained by p110β antibody microinjection (Vanhaesebroeck et al., 1999). Our data are in line with a recent observation that p110β is mainly coupled to GPCR and not to tyrosine-kinase-signalling pathways, and does not contribute to CSF1-stimulated Akt activation in fibroblasts and primary macrophages (Guillermet-Guibert et al., 2008).

Given that the expression levels of the PI3K isoforms are similar in primary and transformed cells (Fig. 7 and data not shown), other mechanisms must underlie this 'switch' in relative importance of PI3K isoforms. Such changes could be intrinsic to the PI3K themselves, such as mutational status (Samuels et al., 2004), or might be due to changes in the cellular environment, such as altered expression or activation of small GTPases that differentially impact on specific PI3K isoforms. These GTPases include Ras and Rab5, which have been shown to differentially interact with class IA PI3K isoforms (Christoforidis et al., 1999; Deora et al., 1998; Rodriguez-Viciana et al., 2004). It is also possible that primary and transformed cells show differential expression of p85 isoforms/splice variants, which could alter p110 isoform function. It is now important to investigate such changes in more defined model systems of cell transformation.

Taken together, our findings clearly show that the relative contributions of p110 isoforms to signalling and biology can differ significantly in primary and immortalised cells. PI3Ks are considered important new pharmacological targets in inflammation and cancer (Hennessy et al., 2005; Rommel et al., 2007; Ward and Finan, 2003; Wymann et al., 2003). The data presented here and in earlier studies (Ali et al., 2004; Ali et al., 2008; Okkenhaug et al., 2002) showing a dominant role of p110δ in primary leukocytes suggest that inhibitors with selectivity for only p110δ may provide excellent opportunities in the intervention of inflammatory and autoimmunity conditions. In cancer, however, a broader inhibition of PI3K isoforms, especially targeting p110α, might be crucial for an optimal clinical outcome.
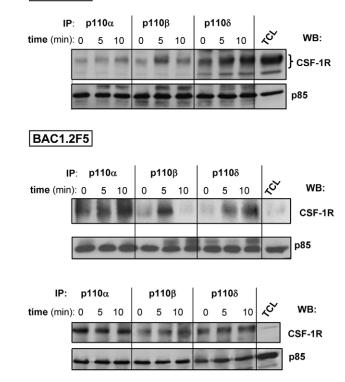
Fig. 7. (A) Expression levels of class IA PI3K isoforms in BAC1.2F5 cells and BMMs (WT or expressing inactive p110α or p110δ) were determined by immunoblotting of total cell lysates (80 μg per lane). (B) Selective recruitment of p110δ to the CSF1R in BMMs but not in BAC1.2F5 cells. WT BMMs or BAC1.2F5 cells were incubated with CSF1 (30 ng/ml) for the indicated times, followed by lysis and immunoprecipitation of p110 isoforms. The co-precipitated CSF1R was detected by western blotting. Equal loading was assessed by reprobing the membrane with antibodies against p85. TCL, total cell lysate (100 μg). For BMMs, a representative experiment out of three is shown. For BAC1.2F5 cells, the results of two independent, representative experiments are shown.

Materials and Methods

Materials
Antibodies against the PI3K subunits used for immunoprecipitation and western blotting have been described (Vanhaesebroeck et al., 1999). Other antibodies and sources were as follows: antibodies to Akt were from New England Biolabs (9272), polyclonal rabbit antibody to CSF1R (sc-692) and monoclonal mouse antibodies to RhoA (sc-418) and PTEN (A2B1) were from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit polyclonal anti-p85 (06-195), monoclonal anti-Rac1 (05-389) were from Upstate Biotechnology. Rac1 activation assay kit [including a GST fusion of the p21-binding domain of PAK1 (GST-PBD) bound to glutathione-Agarose and lysis/wash buffer] were from Upstate Biotechnology. Sources of other reagents were as follows: PTEN ELISA kit (Echelon Biosciences), CSF1 (Peprotech) and ECL Western blotting kit and protein-A- or protein-G-Sepharose (GE Healthcare).

Isolation and culture of BMMs
BMMs were derived from at least three 6- to 8-week-old mice per experiment and pooled. Cells were seeded on bacteriological plastic plates at 10^6 cells/ml in macrophage growth medium consisting of RPMI 1640 (Gibco-Invitrogen Ltd., Paisley, UK), 1 mM sodium pyruvate (Gibco), 1× non-essential amino acids (Gibco), 0.029 mM 2-mercaptoethanol (Sigma), 10% heat-inactivated bovine Ultra low IgG FCS (Gibco) supplemented with 10% L-cell-conditioned medium as a source of CSF1. After 3 days, non-adherent cells were collected and either cryogenically stored in FCS containing 10% DMSO or seeded at 6-8×10^5 cells/ml on bacteriological Petri dishes and cultured for 4 days before use. Cells were detached using EDTA, centrifuged at 1000 *g*, resuspended in macrophage growth medium and seeded for the experiments. All results were obtained from cells that had been cultured for no longer than 10 days after dissection. In all experiments described below (unless otherwise specified) the medium was changed to macrophage starvation medium (macrophage growth medium without L-cell-conditioned medium) 16-20 hours before the actual experiments. Unless otherwise indicated, the concentration of CSF1 for cell stimulation was 30 ng/ml.

BMMs from p110α$^{flox/flox}$ mice, positive or negative for the Cre recombinase, were derived as described above. Cells were seeded for the experiment and 4-hydroxytamoxifen (1 μM; Sigma H790) was added and replaced daily by fresh medium containing tamoxifen. Three to four days later, the cells were harvested for the experiment.

Culture of macrophage cell lines
BAC1.2F5 cells were cultured as previously described (Vanhaesebroeck et al., 1999). Briefly, cells were maintained in growth medium consisting of DMEM, 10% heat inactivated bovine serum, 2 mM L-glutamine, 0.15 mM L-asparagine, 50 nM 2-mercaptoethanol and 10% L-cell-conditioned medium as a source of CSF1. The medium was changed to starvation medium (growth medium without L-cell-conditioned medium) 48-60 hours before the experiments.

J774.2 and IC-21 cell lines were cultured in growth medium consisting of RPMI 1640, 1 mM sodium pyruvate (Gibco), 1× non-essential amino acids (Gibco), 0.029 mM 2-mercaptoethanol (Sigma), 10% heat-inactivated bovine serum and antibiotics. The RAW 264.7 cell line was cultured in DMEM with the same additives as for the J774.2 and IC-21 cells. RAW 264.7 cells were detached with Versene (Gibco). The medium was changed to starvation medium (growth medium containing 5% bovine serum) 20-24 hours before the experiments.

Determination of GTP loading on Rac1 and RhoA
The Rac1 activation assay with GST-PBD (p21-binding domain of PAK, expressed as a GST-fusion protein) was based on the assay method provided by Upstate (www.upstate.com). Cells were lysed in Mg^{2+} lysis buffer, provided in the assay kit, mixed with 8 μg GST-PBD bound to glutathione-agarose and incubated for 1 hour at 4°C. Precipitates were washed three times with Mg^{2+} lysis buffer and suspended in Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE, transferred to PVDF membrane and blotted with anti-Rac1 antibody.

RhoA activation assay was performed using GST-RBD (Rho binding domain of Rhotekin expressed as a GST fusion protein) (Cytoskeleton Inc). Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10% glycerol supplemented with protease inhibitors). Cleared cell lysates were incubated at 4°C for 1 hours with 50 μl glutathione-Sepharose-bound GST-RBD. Precipitates were washed three times with washing buffer (50 mM Tris-HCl pH 7.2, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, supplemented with protease inhibitors) and suspended in Laemmli sample buffer followed by SDS-PAGE and western blotting for RhoA using a monoclonal antibody.

Immunofluorescence microscopy
Cell fixation and direct fluorescence staining of microfilaments by TRITC-phalloidin was done by incubation of cells with 3.7% formaldehyde for 10 minutes, followed by permeabilisation with 0.2% Triton X-100 for 20 minutes. The cells were then incubated for 30 minutes with 2% BSA, followed by TRITC-phalloidin (Sigma) staining of filamentous actin. Slides were mounted using Slow Fade Antifade kit. All specimens were examined on a Zeiss LSM510 confocal laser-scanning microscope, using the accompanying LSM510 software.

Quantification of adhesive area, elongation ratio and ruffling response
Cells were stimulated with CSF1 (30 ng/ml) or vehicle, followed by staining for F-actin as described above. For quantification of cell adhesive area and elongation ratio

(length/breadth), images acquired by confocal microscope were analysed by Image Pro Plus as described previously (Wells et al., 2004). Quantification of ruffling is presented as the percentage of cells with no ruffling, with isolated areas of ruffling covering no more than 50% of the dorsal surface and that with extensive ruffling covering more than 50% of the dorsal surface (relative to the total number of cells observed).

Immunoprecipitation and western blotting

Unless otherwise indicated (in the case of immunoprecipitation for measurements of PTEN activity), cells were lysed in lysis buffer containing 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 50 mM NaF and 1% Triton X-100 supplemented with 10 μg/ml aprotinin, 1 μM pepstatin, 10 μg/ml leupeptin, 1 mM PMSF and 1 mM sodium orthovanadate, followed by clearing of the lysate by centrifugation in a cooled microcentrifuge. Supernatants were directly used for analysis by SDS-PAGE, or immunoprecipitated at 4°C overnight using the indicated antibodies. Immune complexes were collected with 50 μl of 50% slurry of protein-A- or protein-G-Sepharose after incubation for 2 hours, washed with lysis buffer, resolved on 10% SDS-PAGE, and transferred onto PVDF membranes. The blots were probed with the indicated antibodies, followed by detection using enhanced chemiluminescence (Amersham). The band intensities were detected and quantified using an Odyssey infrared scanner (LICOR) using the manufacturer's software.

For assay of PTEN activity, cells were lysed in lysis buffer containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 25 mM β-glycerol phosphate, 1 mM DTT (Lu et al., 2003). Immunoprecipitation was carried out for 90 minutes at 4°C followed by a 2 hour incubation with protein-G-Sepharose.

PTEN lipid phosphatase activity assay

PTEN was immunoprecipitated from 200 μg BMM lysate as described above and then immune complexes were washed twice in lysis buffer, twice in lysis buffer containing 500 mM LiCl and twice in washing buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl). PTEN lipid phosphatase activity was measured on immunoprecipitated PTEN using an ELISA kit from Echelon, according to the manufacturer's instructions. The PIP2 produced was determined, in triplicate experiments, by comparison to a standard curve consisting of PIP2 standards bound to the ELISA plate.

PI3K activity assay

The assays for the determination of IC$_{50}$ (μM) of PW12, TGX155, IC87114 and LY294002 were performed exactly as previously described (Gregan et al., 2007).

[^{3}H]Thymidine incorporation assay

Cells were seeded at 2×10^5 cells per well in 96-well plates, starved for 16 hours or for 48-60 hours in the case of BAC1.2F5 cells, followed by stimulation as indicated in medium containing [^{3}H]Thymidine. 24 or 48 hours later the cells were harvested and the [^{3}H]Thymidine incorporated in DNA measured by scintillation counting.

Transwell migration assay

Cells were seeded on the top chamber of the Transwell (Corning; 55 μm pore size) in the presence or absence of isoform-specific inhibitors and CSF1 (30 ng/ml) was added into the bottom chamber. After 20 hours, the migrating cells on the lower surface of the top chamber were quantified after staining with toluidine blue.

Time-lapse microscopy

Cells were seeded on hydrochloric-acid-washed 22×22 mm coverslips at 2.5×10^4 cells/ml in macrophage growth medium, incubated overnight followed by an 8 hour starvation of CSF1 in macrophage starvation medium. Chemotaxis in Dunn chemotaxis chambers was determined as previously described (Papakonstanti et al., 2007).

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