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Phosphoinositide 3-Kinase p110δ Regulates Natural Antibody Production, Marginal Zone and B-1 B Cell Function, and Autoantibody Responses

Caylib A. Durand,* Karsten Hartvigsen,2† Linda Fogelstrand,2,3† Shin Kim,* Sally Iritani,4‡ Bart Vanhaesebroeck,§ Joseph L. Witztum,† Kamal D. Puri,5‡ and Michael R. Gold5,6*‡

B-1 and marginal zone (MZ) B cells produce natural Abs, make Ab responses to microbial pathogens, and contribute to autoimmunity. Although the δ isoform of the PI3K p110 catalytic subunit is essential for development of these innate-like B cells, its role in the localization, activation, and function of normal B-1 and MZ B cells is not known. Using ICS7114, a highly selective inhibitor of p110δ enzymatic activity, we show that p110δ is important for murine B-1 and MZ B cells to respond to BCR clustering, the TLR ligands LPS and CpG DNA, and the chemokattractants CXCL13 and sphingosine 1-phosphate. In these innate-like B cells, p110δ activity mediates BCR-, TLR- and chemokattractant-induced activation of the Akt prosurvival kinase, chemokattractant-induced migration, and TLR-induced proliferation. Moreover, we found that TLR-stimulated Ab responses by B-1 and MZ B cells, as well as the localization of MZ B cells in the spleen, depend on p110δ activity. Finally, we show that the in vivo production of natural Abs requires p110δ and that p110δ inhibitors can reduce in vivo autoantibody responses. Thus, targeting p110δ may be a novel approach for regulating innate-like B cells and for treating Ab-mediated autoimmune diseases. The Journal of Immunology, 2009, 183: 5673–5684.

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1 Abbreviations used in this paper: MZ, marginal zone; KI, knock-in; LDL, low density lipoprotein; MAA, malondialdehyde-acetaldehyde; MDA, malondialdehyde; OxLDL, oxidized LDL; S1P, sphingosine 1-phosphate; SRBC, sheep RBC.

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develop exhibit impaired chemokine-induced migration, BCR signaling, BCR-induced proliferation, and differentiation into Ab-producing cells (17, 20, 21, 23). Interestingly, mice in which the gene encoding the p110β subunit has been disrupted (p110β−/−) or replaced with a catalytically inactive version (p110βD910A knock-in (KI)) have very few B-1 and MZ B cells (21, 23), indicating that the development of these cells is strongly dependent on p110β. However, because B-1 and MZ B cells are missing in p110β−/− and p110βD910A KI mice, it is not known whether p110β plays a critical role in the function of B-1 and MZ B cells in adult animals. Such studies could provide new insights into the role of p110δ in B-1 and MZ B cell function and reveal whether p110δ inhibitors would be useful therapeutic agents for B cell-mediated autoimmune diseases.

IC87114 is a highly selective inhibitor of p110δ that has been widely used to inhibit p110δ activity in cells that have developed normally in wild-type animals (22, 24–31). It has an IC50 of 0.13–0.5 μM for p110δ (24, 32), whereas its IC50 values for p110α, p110β, and p110γ are at least 200-, 150-, and 60-fold higher, respectively (24, 32). IC87114 does not inhibit cellular processes that have been ascribed to p110δ (33), and it has no inhibitory activity in vitro toward a number of other kinases, including Akt1, PCKα, PKCβII, c-Src, p38 MAPK, casein kinase 1, checkpoint kinase 1, and DNA-PK (24, 34). Unlike the broad-spectrum PI3K inhibitors LY294002 and wortmannin, which act on all cell types, p110δ inhibitors may preferentially block PI3K signaling in B cells where p110δ appears to be the most important isoform.

IC87114 treatment of murine splenic B cells, which are predominantly B-2 B cells, inhibits anti-Ig-induced proliferation, IL-4-dependent survival, and multiple PI3K-dependent signaling events (22), reproducing the effects seen in p110δD910A KI mice. However, this study did not address the role of p110δ in B-1 and MZ B cells, which are important targets for the modulation of Ab-mediated autoimmune diseases.

We now show that inhibiting p110δ activity with IC87114 reduces the migration and activation of B-1 and MZ B cells. Moreover, we show that the in vivo production of natural Abs requires p110δ activity and that treating animals with a p110δ inhibitor can reduce Ab responses in a model of collagen-induced arthritis. Thus, targeting p110δ may be a novel approach for modulating the functions of innate-like B cells in vivo and for treating Ab-mediated autoimmune diseases.

Materials and Methods

Animals, cells, and reagents

C57BL/6 mice and p110δD910A KI mice (p110δD910A/D910A homozygous mice; backcrossed to C57BL/6 for > 10 generations) (23) were used at 6–12 wk of age. Female Lewis rats (7–8 wk old) were obtained from Charles River Laboratories. The University of British Columbia Animal Care Committee (Vancouver, Canada) approved all protocols. Murine splenic B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec). Subsequent FACS sorting was used to enrich CD23−CD21high MZ B cells to > 95% purity (supplemental Fig. 1A). Peritoneal B-1 cells were enriched to > 96% purity (supplemental Fig. 1B) by CD23 depletion and CD19 positive selection using MACS reagents. B cell populations were treated with IC87114 (24), LY294002 (Calbiochem), goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), CXCL13 (R&D Systems), sphingosine 1-phosphate (SIP) (BioMol), LPS (Sigma-Aldrich), lipid A (Avanti Polar Lipids), or CpG DNA (ODN1826; InvivoGen).

ELISA

Mouse serum was diluted in PBS containing 0.05% Tween 20 and added to MaxiSorp 96-well plates (Nunc) coated with 5 μg/ml porcine cardiac myosin (Sigma-Aldrich). 100 μl of mouse heart extract (from M. Horwitz, University of British Columbia), or BSA as a control. Bound Abs were detected with HRP-conjugated goat anti-mouse IgM or IgG and visualized with tetramethylbenzidine (Sigma-Aldrich). Relative Abs0 values were calculated by subtracting BSA control values for each sample. Abs against OxLDL, malondialdehyde (MDA), and malondialdehyde-acetaldehyde (MAA), as well as the E06/T15 Ab, were quantified by chemiluminescent ELISA (35).

Akt phosphorylation

For intracellular staining and phospho-flow analysis, 106 splenic or peritoneal B cells were stimulated in 0.5 ml of modified HEPES-buffered saline (36). The cells were then fixed with 4% paraformaldehyde, permeabilized with 90% methanol for 10 min on ice, blocked with PBS containing 10% donkey serum and 1% BSA, and incubated overnight with a phospho-Akt (Ser473) Ab (Cell Signaling Technologies). Fc receptors were blocked with the 2.4G2 mAb (American Type Culture Collection) and the cells were stained with anti-rabbit IgG-Alexa Fluor 647 (Molecular Probes), anti-CD45R-Pacific blue, anti-CD23-FITC, anti-CD21-PE, and anti-CD5-PECy7 (eBiosciences). Data acquired using a BD Biosciences LSR II flow cytometer was analyzed with FlowJo software (Tree Star).

B cell activation and proliferation

FACS-sorted MZ B cells (106 per well) were cultured in RPMI 1640 with 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM glutamine, and 1 mM pyruvate (complete medium), stained with anti-CD69-PECy7 or anti-CD5-PECy7, and analyzed by flow cytometry. For proliferation assays, total splenic B cells, FACS-sorted MZ B cells, or purified peritoneal B-1 cells (105/well) were labeled with CFSE and then cultured in complete medium (plus 10 mM HEPES for B-1 cells) for 48–72 h. After blocking Fc receptors with the 2.4G2 mAb, cells were stained with 7-amino-actinomycin D and anti-CD45R-Pacific blue or anti-CD19-PE and analyzed by flow cytometry.

Chemotaxis and chemokinesis

Transwell migration assays were performed as described (36, 37), with 106 total mouse splenocytes added to the upper chamber. After 3 h at 37°C, cells that migrated into the lower chamber were stained with anti-CD45R-Pacific blue, anti-CD23-FITC, anti-CD21-PE, and anti-CD5-PECy7 and counted for 30 s using an LSR II flow cytometer. The percentage of migration for each cell type was determined by comparison to the 100% control in which 106 cells were added directly to the bottom chamber.

Rap1 activation

Activated Rap1 that was precipitated using a GST-RalGDS fusion protein, as well as total Rap1 in cell lysates, was visualized by immunoblotting with a Rap1 Ab (Cell Signaling Technologies), as described previously (38). To compare the relative amounts of Rap1-GTP in different samples, band intensities were quantified using NIH ImageJ and the amount of Rap1-GTP was normalized to the amount of total Rap1 in the same sample.

Adhesion assays

Adhesion assays were performed as described (37). MaxiSorp 96-well plates were coated with 30 μg/ml soluble ICAM-1 (Stem Cell Technologies) for 1.5 h and then blocked with BSA. Cells (105) were stimulated in suspension and then added to the wells for 20 min at 37°C. After washing the wells, adherent cells were detached by adding cold RPMI 1640 with 5 mM EDTA for 20 min. Fc receptors were blocked with the 2.4G2 mAb and cells were stained with anti-CD45R-Pacific blue, anti-CD23-FITC, and anti-CD21-PE. The cells were analyzed by flow cytometry, collecting cells for 30 s. The percentage of adhesion for each cell type was determined by comparison to the 100% control in which 105 splenocytes were analyzed directly by FACS.

Oral gavage and immunostaining

C57BL/6 mice were administered 0.1 ml of PEG400 (Hampton Research) or 0.1 ml of 7.5 mg/ml IC87114 (25 mg/kg) in PEG400 twice daily for 9 days by oral gavage. Spleens were preserved in frozen tissue-embedding medium (Fisher) and frozen at −80°C before cutting 7-μm sections using a cryotome (Thermo Electron). The sections were fixed with ice-cold acetone, washed with PBS, and blocked with 1% rat serum and the 2.4G2 mAb for 20 min before being stained with anti-IgM-FITC (Jackson ImmunoResearch Laboratories) and anti-IgD-PE (eBioscience) for 1 h. Sections were washed, mounted with Prolong Gold (Molecular Probes), and imaged using an Olympus FX1000 confocal microscope.
Sheep RBC (SRBC) and collagen Ab responses

Rats were administered IC87114 or vehicle (PEG400) by oral gavage twice daily starting 3 days before they were injected i.p. with 10^8 SRBC and for 10 days afterward, at which time blood was collected. SRBC Abs were measured by ELISA using plates coated with 50 μl of lysed SRBC suspension and HRP-conjugated anti-rat IgM or IgG for detection. For anti-collegen responses, rats were administered IC490194 or vehicle (Suspends-P; Paddock Laboratories) by oral gavage twice daily starting 3 days before they were injected intradermally with 0.2 mg of porcine type II collagen (Chondrex) emulsified in Freund’s incomplete adjuvant. A second intradermal injection of collagen was given on day 14. Blood was collected on days 0 (first collagen injection), 7, 14, 17, and 21. Anti-collagen IgG titers were determined with an ELISA kit (Chondrex) and converted to anticollagen units using a standard curve.

Statistics

For in vitro assays, Student’s two-tailed paired t test was used to compare sets of matched samples. For animal experiments, unpaired t tests were used to determine p values.

Results

IC87114 inhibits PI3K-dependent activation of Akt in splenic and peritoneal B cell subsets

Phosphorylation-dependent activation of the Akt kinase mediates many of the effects of PI3K on cell survival, growth, proliferation, and directional cell migration (39, 40). Therefore, we assessed the role of p110δ in chemokine receptor-, BCR- and TLR-induced activation of Akt in B-1 and MZ B cells from wild-type mice. The chemokine CXCL13 and the lipid chemoattractant S1P regulate B cell trafficking and localization (41, 42), and their receptors activate the PI3K/Akt signaling pathway (36). Intracellular staining and FACS analysis showed that CXCL13 increased the phosphorylation of Akt in splenic B-2, MZ B cells, and B-1a cells and that this response was inhibited, in a dose-dependent manner, by pretreating the cells with the p110δ-selective inhibitor IC87114 (Fig. 1A). IC87114 concentrations of 50–100 nM reduced CXCL13-induced Akt phosphorylation by >50%, and 2 μM IC87114 caused almost complete inhibition of Akt phosphorylation (Fig. 1A, lower panels). IC87114 also inhibited CXCL13-induced Akt phosphorylation in peritoneal B-2 and B-1 B cells (Fig. 1B), as well as S1P-induced Akt phosphorylation in MZ B cells (Fig. 1C). For all of these B cell populations, pretreating the cells with 2–5 μM IC87114 inhibited CXCL13- and S1P-induced Akt phosphorylation to the same extent as the broad-spectrum PI3K inhibitor LY294002. Thus, p110δ-containing PI3K complexes appear to be largely responsible for linking chemoattractant receptors to Akt phosphorylation, even though B cells express all four p110 isoforms.

Clustering the BCR with anti-IgM Abs induced strong Akt phosphorylation in splenic B-2 and B-1a cells, but only a modest 1.3-fold increase in MZ B cells (Fig. 1D). In all cases, both 2 μM IC87114 and 10 μM LY294002 completely blocked BCR-induced Akt phosphorylation (Fig. 1D), indicating that p110δ is the main p110 isoform linking the BCR to Akt phosphorylation in B-2, B-1, and MZ B cells. The TLR9 ligand CpG DNA induced strong Akt phosphorylation in splenic B-2 and MZ B cells, but only a weak response in B-1a cells (Fig. 1E). IC87114 potently inhibited CpG DNA-induced Akt phosphorylation in all B cell subsets, causing nearly complete inhibition at 200 nM and significant inhibition at 20 nM (Fig. 1E, lower panels). Moreover, IC87114 inhibited TLR9-mediated Akt phosphorylation to the same extent as LY294002 (Fig. 1E, upper panels), indicating that p110δ is the main p110 isoform linking TLR9 to Akt phosphorylation in B-2, B-1, and MZ B cells.

p110δ activity is important for TLR-induced proliferation of MZ and B-1 B cells

TLR ligands such as LPS and CpG DNA are key physiological activators of innate-like B cells. We found that LPS and CpG DNA both increased the expression of the early activation markers CD69 and CD86 on purified MZ B cells (Fig. 2A). However, this was not dependent on PI3K because it was not blocked by either the p110δ-selective inhibitor IC87114 or the broad spectrum PI3K inhibitor LY294002 (Fig. 2A). In contrast, IC87114 treatment substantially reduced the ability of TLR ligands to stimulate MZ B cell survival and proliferation (Fig. 2B), consistent with the inhibition of Akt phosphorylation by IC87114. MZ B cells die rapidly in vitro but proliferate vigorously when cultured with LPS or CpG DNA (Fig. 2B). In the presence of 2 μM IC87114, both LPS and CpG DNA-induced MZ B cell survival and proliferation were reduced substantially, as indicated by the decreased numbers of viable cells with reduced CFSE fluorescence (Fig. 2B). IC87114 inhibited CpG DNA-induced MZ B cell survival and proliferation in a dose-dependent manner, with considerable inhibition still evident at 200 nM IC87114 (Fig. 2B). IC87114 treatment also reduced the number of cell divisions that purified peritoneal B-1 cells underwent in response to TLR4 ligands (LPS and purified lipid A) or the TLR9 ligand CpG DNA (Fig. 2C). Thus, p110δ activity is important for both MZ B cells and peritoneal B-1 cells to proliferate in response to TLR ligands.

The relative importance of p110δ in B cell proliferation depended on both the receptor that was stimulated and the B cell subpopulation. For splenic B cells, which are ~80% B-2 cells after MACS isolation, BCR-induced proliferation was most sensitive to inhibition by IC87114, whereas LPS- and CpG DNA-induced proliferation were less sensitive to inhibition of p110δ (Fig. 2D). The differential role of p110δ in different B cell subsets is illustrated by the finding that LPS- and CpG DNA-induced survival and proliferation were more dependent on p110δ activity in MZ B cells (Fig. 2B) than in peritoneal B-1 cells (Fig. 2C) or in splenic B-2 cells (Fig. 2D). Thus, MZ B cells appear to be uniquely sensitive to the inhibition of p110δ activity.

IC87114 inhibits chemoattractant-induced migration of splenic B-2, MZ, and B-1a B cells

CXCL13 and S1P regulate the in vivo localization of B cells. CXCL13 allows circulating B-2 cells to enter lymphoid follicles (43) and is a potent chemoattractant for B-1 cells (43). For MZ B cells, S1P is critical for their retention in the splenic MZ (44) whereas CXCL13 allows MZ B cells to shuttle into the lymphoid follicles, where they deliver blood-borne Ags to B-2 cells (45). Although splenic B cells from p110δ<sup>−/−</sup> KI mice exhibit impaired migration to CXCL13 (17), the role of p110δ in the migration of B-1 and MZ B cells is not known and the ability of IC87114 to modulate the trafficking of these cells has not been investigated.

Using Transwell migration assays, we found that IC87114 treatment reduced CXCL13-induced migration of splenic B-2 cells, B-1a, and MZ B cells by 40–60% (Fig. 3A). IC87114 reduced the migration of these cells to the same extent as LY294002, suggesting that p110δ is the main PI3K isoform that mediates CXCL13-induced migration. IC87114 also inhibited S1P-induced MZ B cell migration by >50% (Fig. 3B). The inhibition of B cell migration by IC87114 and LY294002 was not due to toxicity, because the cells remained viable in the presence of these inhibitors for at least 12 h (data not shown). Moreover, T cell migration toward CXCL12, which is dependent primarily on p110γ (17), was not significantly inhibited by IC87114 (supplemental Fig. 2), indicating that IC87114 does not have off-target effects on p110γ or other...
FIGURE 1. IC87114 inhibits Akt phosphorylation in B-2, B-1, and MZ B cells. A, Total splenic B cells were pretreated with IC87114, LY294002, or an equivalent volume of DMSO (solvent for IC87114 and LY294002) for 30 min and then stimulated with 200 nM CXCL13 for 2 min. FACS analysis was used to quantify intracellular phospho-Akt levels and to distinguish splenic B-2 cells (CD45R^-H11001^-CD23^highCD21^int; where “int” is “intermediate”), MZ B cells (CD45R^-H11001^-CD23^lowCD21^high), and B-1a cells (CD45R^-H11001^-CD5^high). Representative FACS plots are shown. In the corresponding IC87114 dose-response graphs (lower panels), the difference in mean fluorescence intensity values between unstimulated cells and cells stimulated with CXCL13 in the presence of DMSO was used as the 100% value (control response). The difference in mean fluorescence intensity...
enzymes involved in lymphocyte migration. Thus, p110 activity contributes to the ability of innate-like B cells to migrate efficiently toward CXCL13 and S1P.

Chemoattractant-induced migration (chemotaxis) reflects persistent directional movement of cells along the chemoattractant gradient, as well as an increase in the number of motile cells and the speed of their motility. The combined effect of the increased number of motile cells and their increased speed is referred to as chemokinesis, which is independent of directional migration (46). The relative contribution of chemokinesis vs increased persistence of directional movement to a chemotactic response may vary depending on the chemoattractant and the cell type. Moreover, distinct signaling pathways may regulate chemokinesis vs persistent directional migration. Indeed, in neutrophils p110 activity is critical for directional migration along a chemokine gradient but is not required for chemoattractant-induced increases in random cell motility, a feature of chemokinesis (24). If PI3K-independent chemokinesis contributed significantly to CXCL13- and S1P-induced B cell migration, this would account for the partial inhibition of B cell migration by IC87114 and LY294002.

values between unstimulated cells and cells stimulated with CXCL13 in the presence of IC87114 is expressed as a percentage of the control response. Each bar is the mean ± SEM for at least three experiments. B, Peritoneal B-1 cells were pretreated with DMSO, IC87114, or LY294002 for 30 min before being cultured with LPS or CpG DNA (Stim, stimulator) for 12 h. Cell surface expression of CD69 and CD86 was analyzed by flow cytometry. C, Purified MZ B cells were labeled with CFSE. The cells were either analyzed immediately by FACS (time 0) or cultured for 72 h with LPS or CpG DNA in the presence of either DMSO or IC87114 before being analyzed by FACS. Representative FACS plots show the CFSE fluorescence for the viable cells, which were identified by forward and side scatter profiles as well as by low 7-amino-actinomycin D staining. The area under each curve reflects the relative numbers of viable cells at the end of the culture period. D and E, Purified peritoneal B-1 cells (C), or total splenic B-1 cells (D), were labeled with CFSE and either analyzed immediately (time 0) or stimulated with LPS or CpG DNA in the presence of DMSO or IC87114 for 48 h (splenic B cells) or 72 h (B-1 cells). Cells were analyzed as in B. For each panel, similar results were obtained in three experiments.

FIGURE 2. IC87114 inhibits TLR-induced proliferation of MZ B cells and peritoneal B-1 cells. A, Purified MZ B cells were cultured in medium alone for 12 h (unstimulated) or pre-treated with DMSO, 2 μM IC87114, or 10 μM LY294002 for 30 min before being cultured with LPS or CpG DNA (Stim, stimulator) for 12 h. Cell surface expression of CD69 and CD86

emitted proliferation of MZ B cells and peritoneal B-1 cells. A, Purified MZ B cells were cultured in medium alone for 12 h (unstimulated) or pre-treated with DMSO, 2 μM IC87114, or 10 μM LY294002 for 30 min before being cultured with LPS or CpG DNA (Stim, stimulator) for 12 h. Cell surface expression of CD69 and CD86 was analyzed by flow cytometry. B, Purified MZ B cells were labeled with CFSE. The cells were either analyzed immediately by FACS (time 0) or cultured for 72 h with LPS or CpG DNA in the presence of either DMSO or IC87114 before being analyzed by FACS. Representative FACS plots show the CFSE fluorescence for the viable cells, which were identified by forward and side scatter profiles as well as by low 7-amino-actinomycin D staining. The area under each curve reflects the relative numbers of viable cells at the end of the culture period. C and D, Purified peritoneal B-1 cells (C), or total splenic B-1 cells (D), were labeled with CFSE and either analyzed immediately (time 0) or stimulated with LPS or CpG DNA in the presence of DMSO or IC87114 for 48 h (splenic B cells) or 72 h (B-1 cells). Cells were analyzed as in B. For each panel, similar results were obtained in three experiments.
To test this idea, we used a standard approach for assessing chemokinesis, placing CXCL13 in both the upper and lower Transwell chambers such that the cells in the upper chamber were exposed to CXCL13, but there was no gradient. Fig. 3C shows that CXCL13 greatly enhanced chemokinesis in all B cell subsets. The number of cells migrating from the upper chamber to the lower chamber under chemokinesis conditions (indicated by “Both” in Fig. 3C) was 50% of that observed under chemotaxis conditions in which CXCL13 was present only in the bottom chamber. Strikingly, CXCL13-induced chemokinesis was not significantly inhibited by IC87114 (p > 0.05 in all experiments), whereas chemotaxis was reduced to 50% of control values (Fig. 3C), a level that could be attributed to the p110δ-independent chemokinesis. Similar results were obtained with S1P (data not shown). Thus, p110δ activity appears to be critical for CXCL13- and S1P-induced directional B cell migration but is dispensable for the increased chemokinesis caused by these chemotactants. These data also indicate that IC87114 treatment did not down-regulate expression of the receptors for CXCL13 and S1P, because both of these chemotactants stimulated robust chemokinesis in the presence of IC87114.

**p110δ activity is required for activation of the Rap1 GTPase**

We have previously shown that activation of the Rap1 GTPase is essential for CXCL13- and S1P-induced B cell migration, as well as for chemoattractant- and anti-Ig-induced adhesion (36). PI3K activity is required for BCR-induced Rap1 activation (47), but the relevant PI3K isoform has not been identified. Moreover, it is not known whether PI3K activity is required for chemoattractant-induced Rap1 activation or whether TLR ligands induce Rap1 activation in B cells. Fig. 4 shows that CpG DNA induced robust Rap1 activation in splenic B cells. Importantly, we found that treating splenic B cells with IC87114 blocked anti-Ig-, CXCL13-, S1P-, and CpG DNA-induced Rap1 activation (Fig. 4). Thus in B cells, p110δ links the BCR, chemoattractant receptors, and TLRs to the activation of Rap1, a key regulator of B cell migration and adhesion.
IC87114 inhibits MZ B cell adhesion in vitro and disrupts MZ B cell localization in vivo

The retention of MZ B cells in the MZ surrounding the lymphoid follicles of the spleen depends on strong integrin-mediated adhesion (44). Therefore, we asked whether activation of the LFA-1 integrin in MZ B cells was dependent on p110α activity. We found that CXCL13 increased the ability of MZ B cells to adhere to immobilized ICAM-1 (\(p < 0.05\)) and that this LFA-1-dependent adhesion was reduced when the cells were treated with either IC87114 or LY294002 (Fig. 5A). To test whether p110α activity is required for maintaining the in vivo localization of MZ B cells, we treated mice with IC87114 for 9 days and then imaged the organization of B cells in the spleen (Fig. 5, B–G). Spleens of vehicle-treated wild-type mice had thick rings of IgM high IgD low MZ B cells surrounding almost every B cell follicle (Fig. 5, D and F), similar to the spleens of untreated mice (Fig. 5B). In contrast, mice treated with IC87114 had substantially reduced numbers of MZ B cells surrounding the follicles (Fig. 5, E and G), and in many cases the MZ B cells did not completely surround the follicle or were lacking altogether. Although the reduction in MZ B cells in mice treated with IC87114 for 9 days was not as severe as that in the p110αD910A KI mice (Fig. 5C), in which MZ B cells may not develop, these data indicate that p110α activity is important for maintaining the correct localization of MZ B cells in the spleen. Interestingly, the organization of the B-2 cells in the lymphoid follicles (Fig. 5, E and G), as well as the organization of the T cell zone (data not shown), was not affected by IC87114 treatment. Thus, the in vivo localization of MZ B cells in the spleen can be selectively disrupted by IC87114 treatment.

p110α activity is important for normal levels of circulating natural Abs and for TLR-induced Ab responses by B-1 and MZ B cells

MZ and B-1 B cells are major sources of natural Abs that recognize microbial Ags and self-Ags. To assess the role of p110α in the production of natural Abs, we looked at the levels of circulating Abs against oxidation-specific epitopes. These epitopes are found on oxidized lipids and apoptotic cells and are recognized by a large number of natural Abs (7). Consistent with the impaired
development of MZ and B-1 B cells in p110δ-deficient mice, unimmunized p110δ/D910A KI mice had significantly reduced levels of IgM anti-OxLDL Abs in their serum. This was true for IgM natural Abs against copper-OxLDL (A) or MDA-LDL (B) using a chemiluminescent ELISA. Data are reported as relative light units (RLU) per 100 ms (mean ± SD for three mice). Similar results were obtained using MAA-LDL (supplemental Fig. 3A) as well as MDA-BSA and MAA-BSA (supplemental Fig. 3, B and C). There was very low reactivity against native LDL (data not shown) or unconjugated BSA (supplemental Fig. 3D), indicating that the Abs were specific for the MDA and MAA oxidation-associated epitopes. C, A chemiluminescent ELISA was used to quantify the E06/T15 natural Ab in serum from three wild-type and three p110δ KI mice. Dilution curves for sera from individual mice are shown. D, Purified MZ B cells were cultured for 72 h with medium alone or 1 μM CpG DNA in the presence of DMSO or 2 μM IC87114. Culture supernatants were assayed for total IgM. E–G, Purified peritoneal B-1 cells were cultured for 72 h with medium alone, 25 μg/ml anti-IgM, 1 μM CpG DNA, 10 μg/ml LPS, or 1 μg/ml purified lipid A in the presence of DMSO or 2 μM IC87114. Culture supernatants were assayed for total IgM (E) or for IgM Abs specific for copper-OxLDL (F) or MDA-LDL (G). Each bar is the mean ± SD for triplicate samples. Unstim, Unstimulated.
pathogenic Abs by innate-like B cells. Culturing MZ B cells from wild-type mice with IC87114 significantly reduced the ability of CpG DNA to induce the production of cardiac myosin Abs (Fig. 7C). Similarly, the addition of IC87114 to peritoneal B-1 cell cultures reduced the ability of LPS, lipid A, and CpG DNA to induce the secretion of Abs against cardiac myosin (Fig. 7D). Thus, inhibiting p110δ activity with IC87114 can reduce the production of pathogenic self-reactive Abs by innate-like B cells from wild-type animals.

**p110δ inhibitors can reduce pathogenic Ab responses in vivo**

The requirement for p110δ activity in Ab production by both B-2 cells (23) and innate-like B cells (Figs. 6 and 7) suggests that p110δ inhibitors could be used to reduce the production of autoimmune Abs, regardless of which types of B cells produce them. To test this, we initially asked whether administering p110δ inhibitors could reduce in vivo Ab responses to SRBC, a T-dependent Ag. B-2 cells are primarily responsible for T-dependent Ab responses, although MZ B cells can make significant contributions to the early IgM and IgG1 responses to T-dependent Ags (51, 52). We found that treating rats with IC87114 significantly reduced the production of IgM anti-SRBC Abs and, to a lesser extent, IgG anti-SRBC Abs (Fig. 8A). This is the first demonstration that IC87114 treatment can inhibit T-dependent Ab responses in vivo.

We then asked whether p110δ inhibitors could reduce the production of anti-collagen Abs in a rat model of collagen-induced arthritis, an Ab-mediated autoimmune reaction. In this case, we used IC490194, a close structural analog of IC87114. IC490194 is a potent and selective PI3K p110δ inhibitor that has an IC50 of 12 nM for p110δ, 8 µM for p110α, 940 nM for p110β, and 800 nM for p110γ (data not shown). Importantly, IC490194 is metabolized less rapidly than IC87114, and its pharmacokinetic properties are more suitable for chronic dosing of rats. We found that pretreating rats with IC490194 significantly reduced the circulating levels of IgG anti-collagen Abs that were present at 17 and 21 days after collagen injection (Fig. 8B). Preliminary results indicate that IC490194 treatment also caused a significant reduction in joint swelling, bone loss, and the production of proinflammatory cytokines in this collagen-induced arthritis model (J. Douangpanya, S. Iritani, J. S. Hayflick, and K. D. Puri, manuscript in preparation). The ability of IC490194 to inhibit multiple aspects of this complex autoimmune response suggests that p110δ inhibitors may be of therapeutic value for B cell-mediated autoimmune diseases such as rheumatoid arthritis.

**Discussion**

Natural Abs produced by B-1 and MZ B cells protect against microbial infection and carry out homeostatic functions. For example, the T15/E06 IgM natural Ab, which binds phosphocholine moieties attached to the cell wall polysaccharide of many bacteria, protects mice against infection with *Streptococcus pneumoniae* (3, 39). E06 also recognizes the phosphocholine head group of oxidized phospholipids that are present in oxidized lipoproteins and on the surface of apoptotic cells, thereby facilitating the clearance and neutralization of these proinflammatory stimuli (6, 7). Indeed, in mouse models of atherosclerosis, a more severe disease is observed in mice that cannot secrete IgM (53). The p110δ-dependent development of B-1 and MZ B cells appears to be critical for the production of E06, as well as IgM natural Abs against OxLDL, as the circulating levels of these Abs were greatly decreased in p110δ<sup>δ910A</sup> KI mice. Although it is not known whether the decreased levels of natural Abs in p110δ<sup>δ910A</sup> δ KI mice result in...
increased susceptibility to atherosclerosis or \textit{S. pneumoniae} infection, these mice do not appear to be more prone to bacterial infections when housed in conventional mouse facilities. Thus, the levels of circulating natural Abs required for such homeostatic functions remains to be determined.

Innate-like B cells and the Abs they produce have also been implicated in both acute and chronic autoimmune reactions. Hence, the ability to inhibit the functions of innate-like B cells in adult animals could be a useful approach for treating both acute and chronic autoimmune diseases. IC87114 is an inhibitor of p110\(\delta\) (24, 54) that phenocopies many of the effects of disrupting the p110\(\delta\) gene or replacing it with a catalytically inactive version (i.e., p110\(\delta^{D910A}\) KI) (22, 23). Because B-1 and MZ B cells do not develop in p110\(\delta^{-/-}\) mice or p110\(\delta^{D910A}\) KI mice, we used IC87114 to show for the first time that the enzymatic activity of p110\(\delta\) is important for the function of normal B-1 and MZ B cells from wild-type adult mice. We found that p110\(\delta\) signaling contributes to chemokine-induced directional migration as well as TLR-induced proliferation and Ab production by these innate-like B cells. We also show for the first time that the BCR, TLRs, and chemokine receptors activate the Akt prosurvival kinase in B-1 and MZ B cells and that this is completely dependent on p110\(\delta\) activity. Importantly, we show that p110\(\delta\) inhibitors can reduce Ab production by both innate-like and adaptive B cells.

This suggests that such inhibitors could be useful for treating Ab-mediated inflammatory diseases. In support of this idea, we demonstrated that treating animals with p110\(\delta\) inhibitors reduced the in vivo production of proinflammatory autoimmune Abs in a rat model of autoimmune arthritis.

Chemokine-induced migration and adhesion controls the trafficking and localization of B cells, processes that are critical for B cells to encounter Ag and become activated. This is the first report indicating that p110\(\delta\) activity is important for MZ and B-1 cell migration and for MZ B cell adhesion. Importantly, our finding that p110\(\delta\) signaling is important for CXCL13- and S1P-induced activation of the Rap1 GTPase provides a mechanism by which p110\(\delta\) could regulate B cell chemotaxis and adhesion. Rap1 activation is critical for chemokine-induced migration of B cells and adhesion (36), as well as lymphocyte polarization (55), which is essential for directional migration. Akt activation also promotes cell migration via the actin-binding protein girdin (40), and we showed that inhibiting p110\(\delta\) activity completely blocked CXCL13- and S1P-induced Akt activation in B-1 and MZ B cells.

The localization of MZ B cells to the MZ surrounding lymphoid follicles in the spleen positions them to rapidly detect blood-borne microbial infections and depends on counterbalanced gradients of CXCL13 and S1P as well as strong integrin-mediated adhesion (44, 45). We found that treating mice with the p110\(\delta\) inhibitor IC87114 resulted in decreased numbers of MZ B cells surrounding the primary follicles in the spleen. It is not clear whether inhibition of p110\(\delta\) activity leads to MZ B cell death, their migration to other sites, or to a change in surface marker expression such that they can no longer be identified as MZ B cells. However, these findings, together with the observation that Rap1b-deficient mice have greatly reduced numbers of MZ B cells (56), suggest that p110\(\delta\)-dependent activation of Rap1 is important for the proper localization of MZ B cells.

TLR-induced activation can release MZ B cells from the marginal sinus (57) and may allow them to migrate to other sites in response to CXCL13. The trafficking of self-reactive MZ B cells to other organs could contribute to autoimmunity via localized Ab production or via their ability to act as APCs. MZ B cells traffic to secretory glands in Sjogren’s disease (58). Moreover, the migration of MZ B cells to the pancreatic lymph node, where they present self-Ags to autoreactive T cells, has been implicated in the development of diabetes in NOD mice (59). IC87114 effectively blocks the ability of B cells to present Ags and activate T cells (18). These data, together with our findings that IC87114 reduces both CXCL13-induced MZ B cell migration and TLR-induced Ab production by MZ B cells, suggest that p110\(\delta\) inhibitors could reduce the ability of MZ B cells to traffic to other organs and contribute to autoimmune diseases.

Abs made by B-1 and MZ B cells have been implicated in acute inflammatory responses, and reducing the levels of such Abs may have therapeutic utility. Natural Abs against intracellular Ags (e.g., myosins) that are exposed by tissue damage can activate complement, and the resulting inflammation can exacerbate ischemia-reperfusion injury of the heart, mesentery, and brain (5, 8, 9). Our finding that p110\(\delta^{D910A}\) KI mice had significantly decreased titers of natural Abs against heart Ags and cardiac myosin raises the possibility that p110\(\delta\) inhibitors could be useful for reducing the levels of these Abs in normal individuals who have experienced myocardial infarction. We also found that IC87114 treatment reduced the ability of bacterial-derived TLR ligands to stimulate the secretion of Abs to cardiac myosin by B-1 and MZ B cells. Microbial infections have been implicated as a trigger for autoimmunity and, in particular, bacterial infections associated with rheumatic fever induce the production of Abs that cross-react with...
heart tissue and cause heart damage (60, 61). Thus, by inhibiting the activation of innate-like B cells, p110δ inhibitors have the potential to reduce the production of Abs that cause acute inflammatory responses associated with rheumatic heart disease.

Self-reactive Abs made by innate-like B cells also play a major, and sometimes causative, role in chronic autoimmune diseases such as lupus. For example, B cell-specific deletion of the genes encoding the SHP1 protein phosphatase leads to B-1a cell expansion in mice that is accompanied by increased production of self-encoding the SHP1 protein phosphatase leads to B-1a cell expansion. Furthermore, inhibition of p110δ activity could promote B-1 cell expansion. Our demonstration that IC87114 treatment reduced the proliferation of B-1 and MZ B cells, as well as their production of potentially proinflammatory Abs, suggests that the use of p110δ inhibitors could be an important strategy for treating chronic autoimmune diseases.

A key finding was that treating animals with p110δ inhibitors reduced the in vivo production of collagen Abs in a model of collagen-induced arthritis. Because IC87114 inhibits the activation of conventional B-2 cells, as well as B-1 and MZ B cells, the utility of p110δ inhibitors would not be limited to situations in which autoimmunity is mediated by innate-like B cells. The more stringent requirement for p110δ in B cells than in T cells (17, 20), coupled with the restricted expression of p110δ in hematopoietic cells, suggests that p110δ inhibitors could reduce autoimmune Ab production while having minimal effects on other organ systems. Moreover, inhibition of p110δ did not impair the clearance of Staphylococcus aureus in a rat groin abscess model (J. S. Charleston, J. S. Hayflick, and K. D. Puri, manuscript in preparation), suggesting that normal host defense against infections may not be compromised. Thus the use of p110δ-selective inhibitors such as IC87114 may be a new approach for reducing the production of proinflammatory self-reactive Abs in patients with chronic autoimmune diseases and for preventing acute Ab-mediated inflammation and tissue damage after ischemia-reperfusion injury.

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Disclosures
K.D.P. is employed by Calistoga Pharmaceuticals, the maker of potential Abs that make by innate-like B cells are important targets of innate immunity.


