PI3 Kinase δ Is a Key Regulator of Synoviocyte Function in Rheumatoid Arthritis

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Class I phosphoinositide 3 kinase (PI3K) δ is a promising therapeutic target for rheumatoid arthritis (RA) because of its contribution to leukocyte biology. However, its contribution in fibroblasts has not been studied as a mechanism that contributes to efficacy. We investigated the expression and function of PI3Kδ in synovium and cultured fibroblast-like synoviocytes (FLS). Immunohistochemistry demonstrated that PI3Kδ is highly expressed in RA synovium, especially in the synovial lining. Using quantitative PCR and Western blot analysis, we found that PI3Kδ mRNA and protein expression is higher in RA than in osteoarthritis (OA) synovium. PI3Kδ was also expressed in cultured FLS, along with PI3Kα and PI3Kβ, whereas PI3Kγ was not detectable. PI3Kδ mRNA expression was selectively induced by inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) but not by growth factors platelet-derived growth factor (PDGF) and transforming growth factor β (TGFβ). The use of inhibitors that block individual PI3K isoforms, including the novel selective PI3Kδ inhibitor INK007, showed that PI3Kδ is required for PDGF- and TNF-induced Akt activation. PI3Kδ inhibition also diminished PDGF-mediated synoviocyte growth and sensitized cells to H2O2-induced apoptosis. These data are the first documentation of increased PI3Kδ expression in both RA synovium and cultured synoviocytes. Furthermore, these are the first data demonstrating that PI3Kδ is a major regulator of PDGF-mediated fibroblast growth and survival via Akt. Thus, targeting PI3Kδ in RA could modulate synoviocyte function via anti-inflammatory and disease-altering mechanisms. (Am J Pathol 2012, 180:1906–1916; DOI: 10.1016/j.ajpath.2012.01.030)

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Based on these concepts, we studied the expression and function of class I PI3K isoforms in RA synovium and cultured fibroblast-like synoviocytes (FLS), a critical cell that mediates tissue destruction in RA. PI3Kγ protein or mRNA was not detected in synoviocytes. However, we unexpectedly found high expression of PI3Kα in the RA synovial intimal lining and in FLS, where it was induced by pro-inflammatory cytokines and contributed to cell survival and growth. This is the first study showing that this isoform controls functions critical to inflammation in fibroblasts. The data suggest that PI3Kα could play a role in synovial lining hyperplasia as well as the activation and recruitment of inflammatory cells in RA.

Materials and Methods

Preparation of Human Synovial Tissue and FLS

This study was approved by the Institutional Review Board of University of California San Diego School of Medicine, and informed consent was obtained from all participants. Synovial tissue was obtained from patients with OA and RA at the time of total joint replacement or synovectomy, as previously described. The diagnosis of RA conformed to American College of Rheumatology 1987 revised criteria. The samples were either processed for cell culture or snap frozen for immunohistochemistry. For preparation of FLS, the synovium was minced and incubated with 1 mg/mL of collagenase type VIII (Sigma Chemicals, St. Louis, MO) in serum-free RPMI 1640 (Gibco BRL, Grand Island, NY) for 1 hour at 37°C, filtered, extensively washed, and cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products, Calabasas, CA), penicillin, streptomycin, gentamicin, and glucose in a humidified 5% CO2 atmosphere. Cells were allowed to adhere overnight; nonadherent cells were removed, and adherent FLS were split at 1:3 when 70% to 80% confluent. FLS were used from passage 3 through 9, during which time they were a homogeneous population of cells. Cells were allowed to adhere overnight; nonadherent cells were removed, and adherent FLS were split at 1:3 when 70% to 80% confluent. FLS were used from passage 3 through 9, during which time they were a homogeneous population of cells. Cells were allowed to adhere overnight; nonadherent cells were removed, and adherent FLS were split at 1:3 when 70% to 80% confluent. FLS were used from passage 3 through 9, during which time they were a homogeneous population of cells.

Reagents and Antibodies

Cytokines and platelet-derived growth factor BB (PDGF) were obtained from R&D Laboratories (Minneapolis, MN). PI3K inhibitors were provided by Intellikine (La Jolla, CA) with the exception of CAL-101 which was obtained from Selleck Chemicals (Houston, TX) and AS-252424, which was obtained from Caymen Chemical (Ann Arbor, MI). All of the compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L. Concentrations for A66 (PI3Kα), TGX-221 (PI3Kβ), AS-252424 (PI3Kγ) and CAL-101 (PI3Kδ) were chosen based on previous reports. Rabbit monoclonal P-Akt (Ser473), P-Akt (Thr308), pan-Akt, P-GSK3β (Ser9), pan-GSK3β, PI3K p110α, p110β, p110γ, and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal PI3K p110δ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Chemical Synthesis and Inhibitors

INK007 is a potent PI3Kδ inhibitor discovered through structure-based design. INK007 was synthesized with the methods described in the patent (patent no. WO2009088986). Some compounds were synthesized as described in patents (patents WO2004016607, WO2007129161, and WO2009080705).

Immunohistochemistry

Staining protocols were performed as previously described. Cryosections (5 μm) of synovial tissue were cut, fixed in cold acetone for 10 minutes, and incubated with the appropriate Abs overnight at 4°C. Isotype-matched antibodies served as a negative control. Endogenous peroxidase was depleted with 0.3% hydrogen peroxide and sections and then stained with secondary antibodies (Vector Laboratories, Burlingame, CA). The signal was developed using diaminobenzidine and sections were counterstained with hematoxylin.

Western Blot Analysis

Cells were plated in six-well plates, grown until 70% to 80% confluence, and subsequently serum starved (0.1% FBS/DMEM) for 24 hours for synchronization. Cells were washed with cold PBS, and protein was extracted using PhosphoSafe buffer (Novagene, Madison, WI) supplemented with Complete Proteinase Inhibitors (Roche Applied Science, Indianapolis, IN). RIPA buffer was used for protein extraction from synovial tissue. The protein concentrations of tissue and FLS were determined using the Micro BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing 25 μg of protein from cultured FLS or 50 μg of protein from synovial tissue were resolved on Invitrogen NuPage 4% to 12% precast gels and transferred to a PVDF membrane. The membranes were blocked with 5% dry milk, incubated with primary Ab at 4°C overnight, followed by horseradish peroxidase–conjugated secondary Ab for 1 hour. Membranes were developed with Immun-Star Western ECL substrate (Bio-Rad, Hercules, CA) and imaged using the VersaDoc imaging system (Bio-Rad) and QuantityOne software (Hercules, CA) for image capture and densitometry. All Western blot data were analyzed from a single membrane.

Quantitative Real-Time RT-PCR

RNA isolation and RT-PCR were performed as previously described. Forward and reverse primers as well as fluorescent TaqMan FAM/TAMRA-labeled hybridization probes were used (Assays on Demand, Applied Biosystems). To control for sample cellularity, human GAPDH forward and reverse primers and labeled probe were included in separate PCR reactions. The threshold cycle
significant differences for PI3K.

**MTT Assay**

For the MTT assay, 3 x 10³ FLS/well were plated into 96-well plates in 10% FBS/DMEM. After 24 hours, the medium was replaced with low-serum medium (0.1% FBS/DMEM) for 24 hours for synchronization. On day 0, medium was replaced with 1% FBS and cells were treated with PI3K inhibitor at the indicated concentration or with DMSO for 1 hour. PDGF or medium alone was added to the appropriate wells. The experiment was performed in quadruplicate wells. Cell growth was estimated on days 0, 2, 4, 6, and 7 after incubation with MTT for 4 hours and was read at 550 nm with a spectrophotometer.

**Cell Survival and Apoptosis Assays**

For cell survival and apoptosis assays, 3 x 10³ FLS/well were plated onto 96-well plate in 10% FBS/DMEM, then after 24 hours was replaced with starving medium (0.1% FBS/DMEM) for 24 hours for synchronization. Cells were incubated with PI3K inhibitors for 1 hour, then followed by PDGF stimulation. The next day cells were treated with 0.1 mol/L H₂O₂ for 6 hours and viability was determined using an MTT assay. Apoptosis was determined using a Cell Death Detection ELISAPLUS kit (Roche Applied Science, Mannheim, Germany).

**Statistical Analysis**

Statistical analyses were performed using the paired Student’s t-test. A comparison was considered significant if P was less than 0.05.

**Results**

**PI3Kδ Gene and Protein Expression in RA Synovium**

We initially examined PI3K protein expression in synovial tissues using immunohistochemistry. PI3Kδ is highly expressed in the rheumatoid synovium, with especially prominent staining in the synovial lining (representative example in Figure 1A). A similar pattern was observed for PI3Kα (not shown). We next examined relative expression of each PI3K isoform in RA and OA synovial tissues. Using quantitative PCR, we found that PI3Kδ mRNA expression is significantly greater in RA compared with OA (P < 0.05; n = 6 from each; Figure 1B). There was a trend for increased PI3Kγ mRNA in RA, although it did not reach statistical significance (P = 0.06). There were no significant differences for PI3Kα or β mRNA between OA and RA (Figure 1B). Increased PI3Kδ protein expression in RA synovium was confirmed with Western blot analysis, with 1.5-fold higher levels in RA compared with OA (P < 0.04) (Figure 1C).

**PI3Kδ Expression by RA Fibroblast-Like Synoviocytes**

Because PI3Kδ protein is localized to intimal lining cells, we evaluated its expression and regulation in cultured fibroblast-like synoviocytes (FLS). As expected, mRNA for α and β isoforms was detected. Surprisingly, PI3Kδ gene expression was readily detected in all FLS lines, with no difference between RA and OA cells (n = 8 each). mRNA for PI3Kγ was not detected in any FLS line (n = 12 RA and OA) even though control cells were positive (Ramos human B cells, MCP-1 human monocytes, and PBMCs; data not shown) and a previous report noted PI3Kγ in FLS.23 Protein expression for PI3Kδ was confirmed with Western blot analysis (n = 5) (Figure 2A) and immunohistochemistry (Figure 2B). The lack of PI3Kγ in synoviocytes was confirmed with Western blot analysis (n = 5) (Figure 2A) and remained undetected even after overnight stimulation with TNF (see supplemental Figure S1 at http://ajp.amjpathol.org). PI3Kα and β protein was also detected (n = 5) (Figure 2A).

**PI3Kδ Regulation in Cultured FLS**

To determine whether PI3Kδ expression is regulated by inflammatory mediators implicated in RA, we stimulated FLS with tumor necrosis factor (TNF), interleukin-1 (IL-1), platelet-derived growth factor (PDGF), or transforming growth factor β (TGFβ) for 4 to 48 hours and assayed PI3K mRNA using quantitative PCR. PI3Kδ mRNA increased four- to fivefold after TNF or IL-1 stimulation within 6 hours and peaked at 24 hours (Figure 3A for TNF data, n = 4, P < 0.04), whereas PDGF or TGFβ had no effect (data not shown). PI3Kδ induction by TNF or IL-1 was concentration dependent, with maximal effects seen at 50 ng/mL and 2 ng/mL, respectively (Figure 3B). PI3Kα and PI3Kβ mRNA levels were not affected by IL-1 or TNF (Figure 3C).

**Akt Activation in RA FLS**

Akt is a key signaling molecule in the PI3K pathway. Full activation requires phosphorylation at two sites: Thr308 and Ser473.30 To investigate a potential role of PI3Kδ and Akt in synoviocytes, cells were stimulated with cytokines (TNF, IL-1), lipopolysaccharide (LPS), or growth factors (PDGF, TGFβ). P-Akt (Thr308), and P-Akt (Ser473) was induced by TNF or IL-1 was concentration dependent, with maximal effects at 250 ng/mL and 25 ng/mL, respectively (Figure 4A). PI3Kδ protein was undetected in FLS (Figure 4B). P-Akt (Thr308), and P-Akt (Ser473) was detected within 5 minutes after PDGF stimulation and remained elevated at least for 30 minutes. There was a transient and low level of P-Akt detected in response to TNF, IL-1, or lipopolysaccharide, whereas TGFβ had no effect (n = 3) (Figure 4A). GSK3β is another signaling molecule downstream of Akt that is constitutively active
and is inactivated on phosphorylation. P-GSK3β was also detected within 1 minute, peaked at 15 minutes, and decreased by 60 minutes (see Supplemental Figure S2 at http://ajp.amjpathol.org). To evaluate the functional relevance of PI3Kδ induction, we pretreated FLS with TNF and determined whether the cytokine potentiates PDGF responses. As shown in Figure 4B, TNF synergized with low concentration of PDGF and increased Akt phosphorylation.

Figure 1. Expression of PI3K isoforms in RA and OA synovial tissue. A: Immunohistochemistry of PI3Kδ in RA synovium demonstrates prominent staining in the synovial intimal lining, as well as scattered sublining cells. Low- and high-power sections (left and right, respectively, of the pairs) are shown; arrow indicates synovial intimal lining. Serial section stained with an isotype control Ab was the negative control. B: Quantitative PCR was performed to quantify PI3Kα, β, γ, and δ mRNA levels in RA and OA synovial tissue. PI3Kδ was greater in RA than OA (*P < 0.05, n = 6 each for RA and OA). There was a trend for increased PI3Kγ mRNA levels in RA tissue (P = 0.06, n = 6). Data are presented as relative expression units, normalized to GAPDH; values are mean ± SEM. C Western blot analysis and densitometry were performed on synovial tissue lysates and showed higher PI3Kδ protein levels in RA compared with OA. A representative blot of three independent experiments is shown (n = 9 different RA and OA tissues). Data are presented as ratio of arbitrary units for PI3Kδ in RA and OA normalized to β-actin (mean ± SEM, *P < 0.04).

Figure 2. Expression of PI3K isoforms in cultured RA FLS. A: Western blot analysis of total cell lysates from RA FLS (n = 5) for PI3Kα, β, γ, and δ. THP1 and Ramos cell lysates were included as positive control. PI3Kα, β, and δ protein were confirmed, but PI3Kγ was not detected. B: Immunohistochemistry for PI3Kδ in RA FLS lines (n = 3) cultured in chamber slides and stained with anti-PI3Kδ or isotype control Ab.
**PI3Kδ is a Major Regulator of Akt in FLS**

Because PDGF is the most effective inducer of P-Akt, we concentrated our subsequent studies on determining whether PI3Kδ plays a role in PDGF-mediated Akt activation. These studies focused on a panel of inhibitors, including the PI3Kδ inhibitor INK007 (Figure 5A and Table 1). In addition, several other PI3K inhibitors were used at previously reported effective concentrations: panPI3K (GDC-0941), PI3Kδ selective (A66), PI3Kβ selective (TGX-221), PI3Kγ (AS-252424), and broad-spectrum panPI3K/mTOR (LY249002). Cells were pretreated with the inhibitors followed by PDGF stimulation for 30 min-

![Figure 3](image-url)  
**Figure 3.** Regulation of PI3Kδ gene expression in cultured FLS. A: FLS were stimulated with TNF (50 ng/ml) (A) or IL-1β (2 ng/ml) (not shown) for 4 to 48 hours. Quantitative PCR was used to determine relative PI3K gene expression. There was an increase in PI3Kδ mRNA levels after TNF stimulation, which peaked at 24 hours. IL-1 stimulation had a similar effect on PI3Kδ expression (not shown). Data are presented as relative expression units, normalized to GAPDH. B: FLS were stimulated with IL-1β (0.0002, 0.002, or 2 ng/ml) or TNF (0.5, 5.0, or 50 ng/ml), followed by quantitative PCR. PI3Kδ induction by IL-1 and TNF was concentration dependent, with maximal effects seen at 2 ng/ml and 50 ng/ml, respectively. C: Expression of PI3K isoforms in RA FLS at 24 hours after TNF (50 ng/ml) or IL-1β (2 ng/ml) stimulation. There was a four- to fivefold increase in PI3Kδ mRNA levels in response to TNF or IL-1, whereas PI3Kα and PI3Kβ mRNA levels did not significantly change. PI3Kγ mRNA was not detected (not shown). Data are presented as fold induction; values are mean ± SEM. *P < 0.04, n = 3 separate FLS lines.

![Figure 4](image-url)  
**Figure 4.** PI3K and Akt activation in cultured FLS in response to TNF and PDGF. A: FLS were serum starved for 48 hours and then treated with PDGF (10 ng/ml), IL-1β (2 ng/ml), TNF (50 ng/ml), lipopolysaccharide (1 μg/ml), or TGFβ (10 ng/ml) for 0 to 60 minutes. Total proteins were extracted and evaluated by Western blot analysis for P-Akt (Thr308), P-Akt (Ser347), total Akt, and β-actin for loading control. B: FLS were serum starved for 48 hours and then treated with TNF (50 ng/ml) for 24 hours before stimulation with PDGF (0.01 ng/ml) for 30 minutes. Western blot analysis and densitometry were used to determine P-Akt (Ser347), total Akt, and β-actin for loading control (not shown). Data are shown as the ratio of arbitrary absorption units of P-Akt (Ser347) and β-actin normalized to medium alone (mean of two separate experiments is shown).
utes. Western blot analysis showed modest or no effect of the PI3Kα or PI3Kβ inhibitors whereas the pan-PI3K inhibition completely prevented Akt activation (Figure 5A). Unexpectedly, INK007 (α) decreased P-Akt (Thr308) and P-Akt (Ser473) in a concentration-dependent fashion with an EC50 of 500 nmol/L (Figure 5C and see Supplemental Figure S3 at http://ajp.amjpathol.org). Consistent with our observation that PI3Kγ was not detected in FLS, the PI3Kγ inhibitor had no effect on Akt activation. When FLS were treated with a combination of INK007 (PI3Kα) and A66 (PI3Kβ), PDGF-induced Akt phosphorylation was also completely prevented even though A66 alone had a limited effect (Figure 5, C and D). These findings suggest that PI3Kα is a key isoform responsible for PDGF-mediated Akt activation in RA FLS, with PI3Kγ contributing to Akt phosphorylation (Figure 5B). PI3Kγ inhibition did not affect P-GSK3β downstream of Akt (see Supplemental Figure S4 at http://ajp.amjpathol.org) or mitogen-activated protein ki-
Phosphorylation (data not shown). To confirm that the effect of INK007 on P-Akt is due to delta inhibition, we used two other PI3K/H9254 inhibitors with different chemical structures and selectivity profiles: INK055 (PI3K/H9253/H9254) and CAL-101 (PI3K/H9254).24–26 As shown in Figure 5E, both compounds, like INK007, decreased P-Akt induction. Because TNF induces PI3K/H9254 expression and synergizes with PDGF–induced Akt activation, we tested the effect of PI3K/H9254 inhibitors INK007 and CAL-101 on TNF-induced P-Akt. P-Akt induction was completely suppressed, suggesting that PI3Kδ is responsible for Akt activation in response to TNF (Figure 5F). Similar results were obtained when cells were stimulated with IL-1β (data not shown).

**PI3Kδ Regulates FLS Growth**

Akt is a major regulator of cell proliferation and survival in multiple cell types. Therefore, we determined whether PI3Kδ inhibition interferes with cell growth in vitro. FLS were pretreated with INK007 (δ), INK055 (γδ), CAL-101 (δ) or the panPI3K inhibitor and then cultured in the presence of PDGF for 7 days. The PI3Kδ inhibitors decreased cell growth in a concentration dependent manner (Figure 6, A–D). The EC50 for the PI3Kδ inhibitor INK007 is 500 nmol/L, similar to the EC50 for blocking Akt phosphorylation (Figure 6, A and B). This effect was most likely due to an effect on proliferation because there was no change in apoptosis in the PI3Kδ-treated cells as determined by release of cytoplasmic histone-associated DNA fragments (Figure 6E).

**Table 1. Profile of PI3K Inhibitors**

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<th>Compound</th>
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<tr>
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</table>

**IC50s (nM) for inhibition of the catalytic subunits of class I PI3Ks by various compounds based on isolated enzyme assays.**

Figure 6. Effect of PI3Kδ inhibition on PDGF-mediated FLS growth. FLS were cultured in the presence of PDGF (10 ng/mL) with or without pretreatment with INK007 (0.3, 1, 3, and 10 μmol/L), CAL-101 (PI3Kδ) (1, 3, and 10 μmol/L), INK055 (PI3Kγδ) (0.3, 1, and 3 μmol/L), LY249002 at various concentrations, or DMSO for 1 week. Cell number was determined on days 0, 2, 4, 6, and 7 using an MTT assay (see Materials and Methods). A: PI3Kδ inhibitor decreased FLS proliferation in a dose-dependent manner. B and C: Dose response of the PI3Kδ and pan PI3K inhibition of PDGF-induced FLS growth on day 4. Each treatment condition was performed in quadruplicate, and the mean absorbance was used in the final analysis. Data are presented as mean ± SEM relative to arbitrary absorbance units normalized to day 0 samples (n = 3 RA FLS lines, P < 0.05). D: Dose response of the PI3Kδ inhibitors CAL-101 and INK055 inhibition of PDGF induced FLS growth on day 4. Each treatment condition was performed in quadruplicate, and the mean absorbance was used in the final analysis. Data are presented as mean relative to arbitrary absorbance units normalized to day 0 samples (n = 2 RA FLS lines). E: INK007 (1, 3, and 10 μmol/L) does not alter apoptosis in cell growth experiments. FLS were cultured as above to determine whether PI3Kδ decreases PDGF-mediated growth. On day 4, apoptosis was determined by histone-associated DNA fragmentation. There was no effect on apoptosis under these conditions. Control denotes positive control with H2O2-treated cells for comparison.
Regulation of Cell Survival and Apoptosis by PI3Kδ

We then evaluated whether increased PI3Kδ and Akt activation induced by PDGF would protect FLS against oxidative stress–induced apoptosis and whether PI3Kδ inhibition would reverse it. FLS were treated overnight with either PDGF or medium, and then challenged with 0.1 mmol/L H2O2, which are conditions that induce apoptosis rather than necrosis.31 PDGF significantly increased synoviocyte survival nearly twofold (Figure 7A). INK007 reversed the protective effect of PDGF effect on H2O2−induced apoptosis (Figure 7B). These findings suggest that PI3Kδ regulates cell survival downstream of PDGF receptor in FLS.

Discussion

Therapy for RA improved with the introduction of biological agents that target cytokines, B cells, or T cells. However, a significant proportion of patients continue to have active inflammation and disease progression despite optimized therapy.32 As an alternative approach, targeting signaling proteins can potentially modulate several key pathways involved in disease pathogenesis.33,34 Understanding the hierarchy and function of the signaling pathways that regulate inflammation and tissue destruction in RA could lead to effective novel therapeutic agents. Inhibiting the PI3K signaling pathway in RA, including the PI3Kδ isoform, is emerging as potential strategy based on their ability to modulate immune function.1−3,35

Class I PI3Ks are lipid kinases that regulate growth, proliferation, apoptosis, cell adhesion, migration, and metabolism of many cell lineages. They are heterodimers composed of a catalytic subunit p110 and a tightly associated regulatory subunit that controls their activation and subcellular localization. Based on the associated regulatory subunit, class I PI3Ks are further subdivided into class IA (p110α, p110β, and p110δ) and class IB (p110γ). The members of class IA are primarily activated by receptor tyrosine kinases, whereas p110γ is recruited to GPCRs. They exert their biological activities via generation of phosphatidylinositol 3, 4, 5 phosphate (PIP3) at the cell membrane, which serves as a secondary messenger to recruit pleckstrin-homology-domain–containing proteins such as Akt. The intracellular concentration of PIP3 is tightly regulated by PI3Ks and phosphatases such as PTEN, SHIP1, and SHIP2.2,30

The tissue distribution of the individual PI3K isoforms in part defines their distinct biological functions. Although PI3Kα and β are ubiquitously expressed, PI3Kγ and δ are restricted primarily to leukocytes and endothelial cells.5−7 In contrast to the α− and β-deficient mice, which are embryonic lethal, δ− and γ-deficient mice are viable, with distinct immune deficits.8,9,12,36−37 Several studies demonstrated that both isoforms play key roles in innate and adaptive immunity, including lymphocyte signaling, mast cell–mediated allergic response, and activation of neutrophils. For instance, PI3Kδ is the primary PI3K isoform that regulate B−cell development, proliferation, and antibody production.8,9,36 Although PI3Kδ is not required for thymic development, it regulates T cell receptor signaling and T cell differentiation.8,12 Similarly, PI3Kδ contributes to activation and function of neutrophils, macrophages, mast cells, and natural killer cells. Although PI3Kγ is the primary regulator of GPCR-mediated chemokine signaling, PI3Kδ is also important, especially at the later stages of leukocyte infiltration at the site of inflammation. For example, PI3Kδ deficiency results in reduced leukocyte emigration only after prolonged treatment with CXCL2 or TNF.16,17,30−39−41

Defining isoform-specific functions for the PI3Ks by gene deletion in mice or siRNA knockdown in cultured cells has been difficult, in part because the changes in expression and function of the remaining isoforms occur, as well as incomplete knockdown (data not shown).42,43 PI3Ks have kinase-independent functions as well. The murine PI3Kδ kinase inactive knock-in but not the p110δ knock-out mice exhibit impaired T cell activity, possibly because of the role that the enzymes play as scaffold proteins.2,44 The recent development of small molecule inhibitors with increased selectivity provides a valuable tool to overcome the limitations of genetic approaches with this particular pathway.2,39,40 This approach was used for the present study, and the use of multiple compounds with distinct structures suggests that the effects are due to PI3Kδ inhibition rather than off-target effects. Like many kinases, the reasons for cell-specific differences in function, despite similar expression levels, are not well defined, and could relate to subcellular

Figure 7. Effect of PI3Kδ inhibition on PDGF-mediated synoviocyte apoptosis in response to oxidative stress. A: FLS were treated with PDGF (10 ng/mL) or medium overnight and then exposed to 0.1 mmol/L H2O2 for 6 hours. Cell viability was determined using an MTT assay. B: Dose-dependent reversal of PDGF effect on cell by PI3Kδ inhibition. Cells were pretreated with INK007 (1, 3, and 10 μmol/L) or DMSO for 1 hour before PDGF and H2O2 treatment as described above. Each treatment was performed in quadruplicate, and the mean absorbance was used in the final analysis. Cell death rate was calculated using MTT viability assay. Data are presented as relative absorbance units, normalized to medium alone and presented as mean ± SEM (n = 3 RA FLS lines)
distribution or the scaffold proteins that bring signaling complexes together. For example, the relative contributions of MKK3 and MKK6 in the p38 pathway probably is due to the nonenzymatic functions of MKK6 as a scaffold in FLS.45

PI3Kγ and PI3Kδ have related but nonredundant functions in leukocytes. The two isoforms, either alone or in combination, are promising targets; but the most effective strategy for a particular autoimmune disease is not known. Studies using knockout or kinase-inactive mice and selective inhibitors of PI3Kδ, PI3Kγ, and PI3Kγδ have been valuable in dissecting the precise role of individual PI3K isoforms in disease models.1,46–51 In the K/BxN serum transfer arthritis model, either PI3Kδ or PI3Kγ blockade decreased disease severity, whereas dual inhibition was synergistic.51 Despite these observations, there is very little known about the specific role of the PI3Kδ in fibroblasts and how it might contribute to the pathogenesis of RA. The fact that P-Akt is higher in RA synovial tissue and FLS compared with OA suggests that this pathway is activated in RA.52

Our initial studies showed that PI3Kδ is highly expressed in rheumatoid synovial tissues at the protein and the mRNA levels. Because PI3Kδ levels were especially high in the synovial intimal lining, we examined its expression and function in cultured synoviocytes. Abundant PI3Kδ expression, along with the α and β isoforms, was found in all FLS lines. The detection of the δ isoform in FLS was unexpected because its distribution is generally restricted to hematopoietic cells. A few exceptions have been noted in the past, most notably in smooth muscle cells, lung fibroblasts, and cancer cell lines.53–56 PI3Kδ mRNA expression in synoviocytes increased several fold after stimulation with pro-inflammatory cytokines like TNF or IL-1. Induction of PI3Kδ expression has only been previously reported in smooth muscle cells after insulin or angiotensin II treatment.53 Although TNF is not a potent activator of PI3K/Akt pathway in FLS, it primed the cells for subsequent robust responses to PDGF even at low concentrations. Using two PI3Kδ selective inhibitors, we also showed that PI3Kδ mediates Akt activation by PDGF, TNF, or IL-1. The effect on TNF function is particularly intriguing, because it suggests that blocking the PI3Kδ pathway might contribute to the benefit of TNF blockers in RA.

PI3Kγ mRNA or protein was not detected in the more than 20 FLS lines evaluated, although a previous report suggested that it can be expressed by RA but not OA FLS.29 The reasons for different results are not certain, but the inclusion of later-stage patients in our population could potentially have contributed. Some disparities ascribed to patient selection have been noted in the past when evaluating p53 mutations in RA FLS.57 The fact that a PI3Kγ-selective inhibitor had no effect on PDGF-induced Akt phosphorylation supports the notion that this isoform is not functionally relevant in our studies.

To explore PI3Kδ function, we used selective PI3K inhibitors designed through computational chemistry to dissect the contribution of individual isoforms. These studies showed that PI3Kδ plays a major role regulating PDGF-mediated Akt activation in FLS. INK007 is a novel PI3Kδ inhibitor, with about 30-fold selectivity over PI3Kγ and greater than 1000-fold selectivity over α or β. The effect of the compound seen in FLS, as well as INK055, is most likely due to inhibition of the δ isoform, because neither mRNA nor protein for PI3Kγ was detected, and a separate PI3Kγ selective inhibitor had no effect. Furthermore, PI3Kγ is mainly activated via GPCRs, and it is unlikely that γ participates directly in PDGF receptor or receptor tyrosine kinase signaling. Specificity for PI3Kδ is also supported by the observation that GSK3β phosphorylation was not affected by INK007 but was decreased by the pan-PI3K inhibitor. This is also consistent with lack of GSK3β inactivation seen in PI3Kδ kinase–inactive mice.58 Unanticipated off-target effects can occur with any chemical compound. To minimize the likelihood that off-target effects account for our observations, we used two additional PI3Kδ inhibitors, CAL-101 and INK055. These compounds have biological effects similar to those of INK007.

PI3Kδ and PI3Kα participate in PDGF-mediated Akt activation in synoviocytes, based on studies with the selective inhibitors. INK007 had the most prominent effect, whereas the PI3Kα inhibitor had less effect but completely blocked Akt phosphorylation in combination with INK007. The interplay between various isoforms has been observed in other situations to maximize cellular response, such as PI3Kα and PI3Kδ in insulin signaling, and PI3Kδ and PI3Kγ for neutrophil reactive oxygen production.22,59 The 500 nmol/L EC50 for INK007 is somewhat higher than expected, based on the isolated enzyme, and might reflect cellular penetration of the compound.

Because the Akt pathway is a major regulator of growth and survival,52,60–62 we studied the effect of PI3Kδ inhibition on synoviocytes growth and apoptosis. PI3Kδ inhibition decreased PDGF-mediated Akt phosphorylation and regulated FLS growth, without significant change in apoptosis under basal conditions. These findings suggest that the change in cell growth is most likely due to an effect on cell proliferation. PI3Kδ inhibition does, however, sensitize cells to H2O2-induced apoptosis in the presence of PDGF, most likely via Akt-dependent pathways.

Our findings extend the biology of PI3Kδ observed in bone marrow–derived cell lineages. For instance, PI3Kδ is required for B-cell proliferation to anti-IgM, anti-CD40, IL-4, or stimulation with TLR ligands.8,9,38 PI3Kδ inactivation also partially impairs T cell clonal expansion and CSF-1–induced DNA synthesis in macrophages.17,63 Overexpression of PI3Kδ contributes to abnormal growth of human acute myeloid leukemia cells and a subset of primary neuroblastomas, suggesting that it might contribute to the transformed phenotype of malignant cells.54,64 However, a dominant biological function of the PI3Kδ over the other isoforms has not been previously described for fibroblasts.

In conclusion, this is the first published report demonstrating that PI3Kδ expression is increased in RA synovium. In contrast to its expected restriction to bone marrow–derived cells, it is abundantly expressed and inducible in cultured FLS. The δ isoform contributes to the growth and survival of FLS and could play a role in synovial hyperplasia in the rheumatoid joint. Targeting
PI3Kδ targeting has previously focused on its effects on innate and adaptive immunity, especially B cell, neutrophil, and mast cell activation. Our findings suggest that targeting PI3Kδ would be not only anti-inflammatory but would protect cartilage by modulating synovioocyte function.

Note Added in Proof

After acceptance of this manuscript, an additional paper also reported expression and induction of PI3KD in cultured synoviocytes.65

References


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