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Phosphoinositide 3-Kinase δ Regulates Migration and Invasion of Synoviocytes in Rheumatoid Arthritis

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Cartilage destruction mediated by invasive fibroblast-like synoviocytes (FLS) plays a central role in pathogenesis of rheumatoid arthritis (RA). Increased cell migration and degradation of extracellular matrix are fundamental to these processes. The class I PI3Ks control cell survival, proliferation, and migration, which might be involved in cartilage damage in RA. PI3Kδ isoform was recently identified as a key regulator of FLS growth and survival, suggesting that it could contribute to synoviocyte aggressive behavior. Therefore, we assessed the role of PI3Kδ in RA synoviocyte migration and invasion. We observed that PI3Kδ inhibition or small interfering RNA knockdown decreased platelet-derived growth factor (PDGF)–mediated migration and invasion of FLS. We then showed that PI3Kδ regulates the organization of actin cytoskeleton and lamellipodium formation during PDGF stimulation. To gain insight into molecular mechanisms, we examined the effect of PI3Kδ inhibition on Rac1/PAK, FAK, and JNK activation. Our studies suggest that Rac1/PAK is key target of PDGF-mediated PI3Kδ signaling, whereas FAK and JNK are not involved. Thus, PI3Kδ contributes to multiple aspects of the pathogenic FLS behavior in RA. These observations, together with previous findings that PI3Kδ regulates FLS growth and survival, suggest that PI3Kδ inhibition could be chondroprotective in RA by modulating synoviocyte growth, migration, and invasion. ***The Journal of Immunology*****, 2014, 192: 2063–2070.**

Rheumatoid arthritis (RA) is an immune-mediated disorder that leads to chronic inflammation and progressive destruction of diarthrodial joints. Fibroblast-like synoviocytes (FLS) in the synovial intimal lining are major effectors of cartilage destruction in this process. In addition, recent observations suggest that FLS promote disease progression by migrating to the unaffected joints. Cartilage invasiveness and migratory phenotype, in addition to increased proliferation and resistance to apoptosis, are unique features of RA synoviocytes. However, underlying molecular mechanisms that regulate this pathogenic phenotype are poorly understood (1–3).

Migration and invasion are complex processes that require dynamic interactions between cells and the surrounding matrix. This coordinated program is executed by multiple pathways that integrate signals from the extracellular environment and remodel the actin cytoskeleton (4, 5). The PI3K family of lipid kinases regulates many important cellular events, including cell migration. The three members of the class IA subgroup (PI3Kα, β, and δ) are generally activated by receptor tyrosine kinases, whereas the class IB PI3Kγ signals through G protein–coupled receptors. PI3Ks control overlapping and nonredundant functions ranging from cell growth and proliferation to migration and cytokine expression (6, 7). Perturbing this signaling cascade is implicated in inflammatory and autoimmune disorders as well as cancer (8–11).

PI3Kδ has captured attention based on its predominant expression in hematopoietic cells, which could minimize toxic effects on other cell lineages. We recently discovered that PI3Kδ is highly expressed in the RA synovial intimal lining and in FLS. PI3Kδ gene expression, unlike the other isoforms, is also induced by inflammatory cytokines in cultured synoviocytes. PI3Kδ serves as a major regulator of platelet-derived growth factor (PDGF)–mediated synoviocyte growth and survival and could therefore contribute to synovial lining hyperplasia (12).

In this study, we determined whether PI3Kδ is also involved in FLS motility and observed that PI3Kδ inhibition or small interfering RNA (siRNA) knockdown decreased migration and invasion of FLS. We then showed that PI3Kδ regulates the organization of actin cytoskeleton and lamellipodium formation through activation of Rac1. Thus, PI3Kδ contributes to multiple aspects of the pathogenic FLS behavior in RA. Inhibition of PI3Kδ might be chondroprotective in RA by modulating synoviocyte function.

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 osteoarthritis (OA) and RA at the time of total joint replacement or synovectomy, as previously described (13). The diagnosis of RA conformed to American College of Rheumatology 1987 revised criteria (14). The synovium was minced and incubated with 1 mg/ml collagenase type VIII (Sigma-Aldrich, St. Louis, MO) in serum-free RPMI 1640 (Life Technologies, Grand Island, NY) for 1 h at 37°C, filtered, extensively washed, and cultured in DMEM (Life Technologies) supplemented with 10% FBS (Gemini Bio Products, Calabasas, CA), penicillin, streptomycin, gentamicin, and glutamine in a humidified 5% CO_2 atmosphere. Cells were allowed to adhere overnight, nonadherent cells were removed, and adherent FLS were split at 1:3 when 70–80% confluent. FLS were used from passages 3 through 9, during which time they are a homogeneous population of cells (<1% CD11b positive, <1% phagocytic, and <1% FcγRII and FcγRIII receptor positive). The detailed characterization and phenotyping of FLS were previously described (15–19). FLS were cultured and

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Abbreviations used in this article: FLS, fibroblast-like synoviocyte; GTPase, guanosine triphosphatase; OA, osteoarthritis; PDGF, platelet-derived growth factor; PDGF-BB, dimeric platelet-derived growth factor composed of two B (-BB) chains; RA, rheumatoid arthritis; siRNA, small interfering RNA.

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used at 80% confluence. Cells were synchronized in 0.1% FBS containing media for 24 h before the addition of the appropriate stimulus.

Reagents and Abs

The dimeric platelet-derived growth factor composed of two B (-BB) chains (PDGF-BB) was obtained from Sigma-Aldrich. PI3K inhibitors were provided by Infinity Pharmaceuticals (Cambridge, MA), CAL-101 was obtained from Selleck Chemicals (Houston, TX), and AS-252424 was obtained from Caymen Chemical. INK007 is a highly selective PI3Kδ inhibitor discovered through structure-based design. It was screened against >400 individual kinases, and its synthesis and properties were described in patent WO2009088986. The IC_{50} for INK007 based on isolated enzyme assays are 0.3 nM for PI3Kδ, 7.4 nM for PI3Kγ, 347 nM for PI3Kβ, and 1093 nM for PI3Kα. The K_D values for IPI-145 are 0.023 nM for PI3Kδ, 0.24 nM for PI3Kγ, 1.56 nM for PI3Kβ, and 25.9 nM for PI3Kα (20). All of the compounds were dissolved in DMSO at 10 mM. Concentrations for A66 (PI3Kα), TGX-221 (PI3Kβ), AS-252424 (PI3Kγ), and CAL-101 (PI3Kδ) were chosen based on previous reports. Anti–phospho-FAK (T397), anti–phospho-FAK (T925), anti–phospho-JNK (T183/T185), anti-JNK, anti–phospho-PAK1/2, anti-PAK1/2, and secondary Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti-FAK (C-20) and anti-PI3K p110δ Ab were obtained from Santa Cruz Biotechnology (Dallas, TX).

Cell migration assays

For the wound closure motility assay, FLS were plated in six-well plates at 70–80% confluence and serum starved (0.1% FBS/DMEM) overnight. A linear wound was created using a 1-ml micropipette tip and then washed three times with starving media to remove unattached cells. Cells were incubated with PI3K inhibitors or DMSO for 1 h, and then 0.1% FBS containing media ± PDGF-BB (10 ng/ml) was added. Light microscopy images were taken immediately 0 and 36 h after wounding. At the end of the experiment, cells were fixed and stained using Hemacolor staining kit (EMD Millipore, Billerica, MA). Light microscope images for three locations of marked wound were taken, and migrated cells were counted using NIH ImageJ software. The number of migrated cells was normalized to media control, and this value represents the migration index.

The directed chemotaxis assay was performed using transwell filters (Corning) with 8-μm pores coated with BSA. Cells were serum starved (0.1% FBS/DMEM) overnight prior to setting up the assay. A total of 2.5×10^4 cells was plated in the top chamber with PI3K inhibitors or DMSO and was allowed to migrate for 6 h toward 0.5% BSA containing media ± PDGF-BB (10 ng/ml) in the lower chamber. After the nonmigrated cells were removed with a cotton swab, the membranes were fixed and stained with crystal violet. The number of migrated cells was averaged from three ×10 field-of-view images and normalized to control.

For the modified spheroid migration assay, FLS were trypsinized and resuspended at a concentration of 1×10^6 cells/50 μl in DMEM. Cells (2 μl) were mixed at 1:1 in 4 mg/ml growth factor–reduced BD Matrigel Matrix (BD Biosciences), pipetted as a spot in a 24-well tissue culture dish, and incubated at 37°C for 15 min to gel (21). Medium 0.1% FBS/DMEM with or without PDGF-BB (10 ng/ml) in the presence of PI3K inhibitors or DMSO was added. Cell movement concentrically was monitored 0–36 h. At the end of the experiment, cells were fixed and stained using Hemacolor staining kit (EMD Millipore). Each spot was imaged by taking four images, and migrated cells were counted using NIH ImageJ software. The number of migrated cells was normalized to media control, and this value represents the migration index.

Invasion assay

The BD BioCoat Growth Factor Reduced Matrigel Invasion Chambers (8-μm pore diameter; BD Biosciences) were used to evaluate invasion through a Matrigel layer. To measure cell invasion, 2.5×10^4 cells in medium containing 0.1% BSA were added to the transwells in the presence of PI3K inhibitors or DMSO. Medium supplemented with PDGF-BB (25 ng/ml) was used as an attractant in the lower chamber. After 24 h, the cells that invaded through the matrix were fixed and stained with Hemacolor staining kit (EMD Millipore). The number of invading cells was averaged from three ×10 field-of-view images and normalized to control.

Immunofluorescence studies

Cell were plated into eight-well chamber slides coated with poly(L-lysine) and serum starved for 24 h. Cell were pretreated with PI3K inhibitors or DMSO for 1 h, followed by PDGF-BB (10 ng/ml) stimulation. Slides were rinsed with PBS, fixed in 3.7% formaldehyde/PBS for 20 min, and permeabilized with 0.1% Triton X-100/PBS for 10 min. Slides were blocked for 1 h with 1% BSA/PBS, and F-actin was visualized with rhodamin-phalloidin (Invitrogen, Eugene, OR) for 30 min. Slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Confocal images were collected using a laser-scanning confocal microscope (Fluoview FV-1000; Olympus) using a ×40 oil immersion objective and were captured into Fluoview software. Image analysis was performed using NIH ImageJ.

Rac1 guanosine triphosphatase activity assay

Activated Rac1 was detected using Rac1 assay reagent (GST-PAK1-PBD on glutathione-Sepharose Beads), according to the manufacturer's instructions (Cytoskeleton, Denver, CO). GST-PAK1-PBD specifically binds to the GTP-bound form of Rac1. RA FLS were plated in 10-cm dishes at 50–70% confluence, serum starved with DMEM for 48 h. Cell were pretreated with PI3K inhibitors or DMSO for 1 h, followed by PDGF-BB (10 ng/ml) stimulation. Samples were processed according to the manufacturer's instructions.

Rac1-GTP and total Rac1 protein levels were visualized by Western blotting.

Western blot analysis

Cells were plated in six-well plates, grown until 70–80% confluence, and subsequently serum starved (0.1% FBS/DMEM) for 24 h 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). The protein concentrations of FLS were determined using the Micro BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples containing 25 μg protein from cultured FLS were resolved on Invitrogen (Carlsbad, CA) NuPage 4–12% precast gels and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% dry milk and incubated with primary Ab at 4°C overnight, followed by HRP-conjugated secondary Ab for 1 h. Membranes were developed with Immun-Star Western ECL substrate (Bio-Rad, Hercules, CA) and imaged on VersaDoc imaging system (Bio-Rad), using QuantityOne software (Hercules, CA) for image capture and densitometry. All Western blot data were analyzed from a single membrane.

siRNA transfection

A total of 5×10^5 FLS (passages 4–6) was transfected with 1–3 μg targeting the p100δ, cataLU of PI3Kδ, or scramble control Smartpool siRNA (Dharmacon, Lafayette, CO), using normal human dermal fibroblast Nucleofector kit, according to the manufacturer's instruction (Amaxa, Gaithersburg, MD).

Statistical analysis

Statistics were performed using the paired Student *t* test. A comparison was considered significant if $p < 0.05$.

Results

PI3Kδ regulates FLS migration

Cell migration is regulated in part by growth factors, such as PDGF, that trigger the motility machinery of cells when applied in a uniform concentration (22). RA FLS migration was first assessed in a wound-healing assay in presence or absence of PDGF-BB. Cell movement into the wounded area was measured with light microscopy (see *Materials and Methods*). To investigate the role of PI3Ks, the assay was performed in the presence or absence of PI3K inhibitors: pan (GDC-0941), PI3Kα (A66), PI3Kβ (TGX-221), PI3Kγ (AS-252424), PI3Kδ (INK007 and CAL-101), and PI3Kδ/γ (IPI-145). As shown in Fig. 1, the number of cells that migrated increased by 2.6-fold in response to PDGF compared with low serum media (0.1% FCS). As expected, the pan-PI3K inhibitor suppressed migration at baseline and in response to PDGF (Fig. 1). Selective PI3Kα and PI3Kδ inhibitors were also effective, with PI3Kδ playing a predominant role (Fig. 1A). PI3Kβ and PI3Kγ inhibitors had no effect (data not shown). The inhibitory effect of PI3Kδ inhibitor (INK007) was dose dependent with 60 ± 5% inhibition at 1 μM ($p < 0.04$) (Fig. 1B). Consistent with our previous observation that the γ isoform is not detected in FLS (12), the effect of a dual PI3Kδ/γ (IPI-145) inhibitor was similar to the selective δ inhibitor. These observations suggest that PI3Kδ activity is

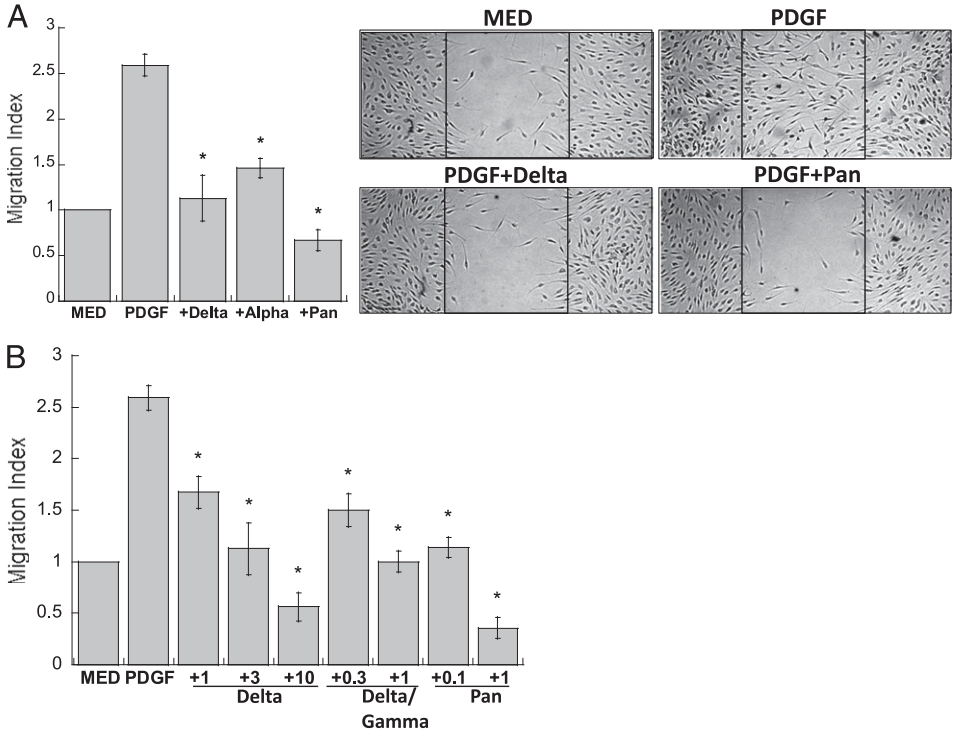

FIGURE 1. PI3K inhibition decreases RA FLS migration in response to PDGF. (**A**) Directed cell migration was analyzed using a wound-healing assay. Wounds were introduced on the surface of confluent six-well dishes following overnight serum starvation. After washing, cells were cultured in low-serum MED (0.1% FCS) alone or with PDGF-BB (10 ng/ml). Light microscopy images were obtained immediately or 36 h after wounding. The number of migrated cells was averaged from six ×4 field-of-view images. Representative light microscopy images of RA FLS monolayers 36 h after wounding are shown. To test the effect of PI3K inhibition, cells were treated with 3 μM PI3Kα (A66), 3 μM PI3Kδ (INK007), and 1 μM pan (GDC-0941) or DMSO. (**B**) Inhibitory effect of PI3Kδ and δ/γ inhibition on RA FLS migration. Cells were treated with INK007 (PI3Kδ) (1, 3, and 10 μM) or IPI-145 (PI3Kδ/γ) (0.3 and 1 μM) or GDC-0941 (panPI3K) (0.1 and 1 μM) or DMSO in MED (0.1% FBS) alone or supplemented with PDGF-BB (10 ng/ml). Original magnification ×40. The migration index represents the number of migrated cells normalized to media control. Data are presented as mean ± SEM ($*p < 0.04$, $n = 6$ RA lines).

required for synoviocyte migration in response to growth factor stimulation.

PI3Kδ knockdown decreases PDGF-mediated synoviocyte migration

To confirm that FLS motility depends on PI3Kδ activity, gene knockdown was also performed with siRNA targeting the catalytic subunit p110δ. The efficiency of the knockdown was confirmed by immunoblotting (Fig. 2A). Cells with reduced p110δ levels displayed significantly lower PDGF-mediated cell migration compared with scramble control (Fig. 2B).

Regulation of directed migration by PI3Kδ

We then tested the effect of PI3Kδ inhibition on chemotaxis in a modified Boyden chamber system using PDGF as a chemoattractant. Transwell membranes were coated with BSA instead of extracellular matrix proteins to assure that we were evaluating the role of PI3Kδ rather than integrin-mediated haptotaxis. INK007 decreased RA

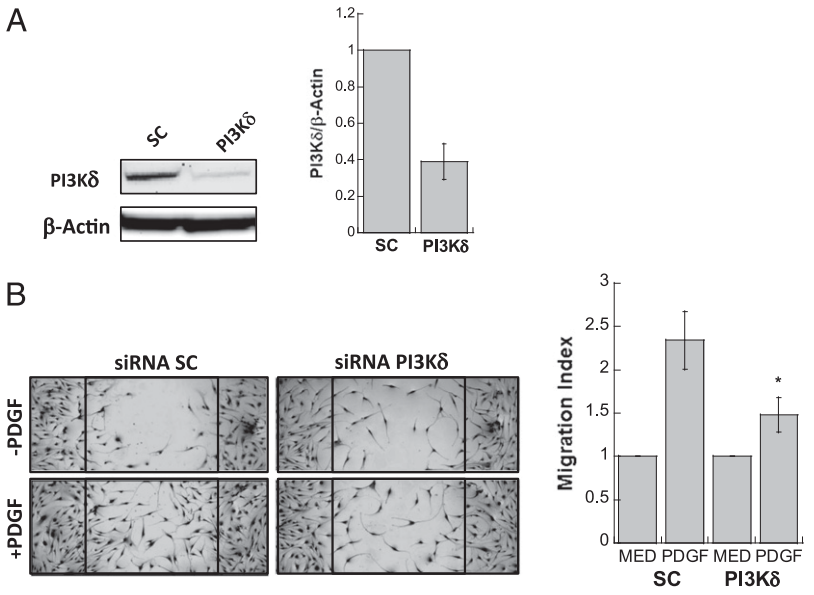

FIGURE 2. Targeted depletion of PI3Kδ impairs RA FLS migration. (**A**) RA FLS were transfected with siRNAs specific for p110δ, the catalytic subunit of PI3Kδ, or control siRNA (SC). After 72 h, cells were lysed, and expression of p110δ was determined by Western blot analysis. Expression of p110δ was quantified by densitometry relative to β-actin. Data are shown as the ratio of arbitrary absorption units of PI3Kδ and β-actin (mean ± SEM, $n = 3$ RA FLS lines). (**B**) RA FLS cells transfected with scramble control or p110δ siRNA were serum starved overnight at 48 h. Cell migration was analyzed 72 h after transfection using wound-healing assay. Representative light microscopy images of RA FLS monolayers 36 h after wounding are shown. Original magnification ×40. The migration index represents the number of migrated cells normalized to media control. Data are presented as mean ± SEM ($*p < 0.03$, $n = 3$ RA lines).

FLS movement by 70% ($p < 0.05$), similar to the maximal inhibition seen with the panPI3K inhibitor (GDC-0941) (Fig. 3A).

Regulation of FLS migration through matrix by PI3Kδ

To mimic the three-dimensional environment, RA FLS were embedded in Matrigel and evaluated for active migration out from the matrix. The matrix was cultured in low serum medium in the presence or absence of PDGF for 48 h. Cell movement was significantly decreased in the presence of PI3Kδ (INK007 and CAL-101) or PI3Kδ/γ (IPI-145) selective inhibitors (Fig. 3B). These results confirm that PI3Kδ inhibition effectively decreases RA FLS–directed migration.

PI3Kδ inhibition impairs RA FLS invasion

The ability to invade cartilage is a key pathogenic behavior of rheumatoid synoviocytes. Therefore, we tested the effect of PI3Kδ inhibitors on RA FLS invasion through Matrigel-coated transwell membranes using PDGF as a chemoattractant. FLS were pretreated with INK007 (PI3Kδ), CAL-101 (PI3Kδ), IPI-145 (PI3Kδ/γ), or GDC-0941 (panPI3K) and cultured in Matrigel invasion chambers for 16 h. The PI3Kδ inhibitors decreased invasion by 50–60% ($p < 0.04$) compared with vehicle-treated control (Fig. 4A) with concentration-dependent manner (Fig. 4B). As expected, panPI3K inhibition also decreased Matrigel invasion of RA FLS (Fig. 4A).

PI3Kδ mediates PDGF-induced actin cytoskeletal changes in RA FLS

PDGF induces cell migration in part through dynamic reorganization of the actin cytoskeleton (23, 24). To visualize actin remodeling in FLS, cells were fixed and stained with rhodamin-phalloidin at 0–90 min following PDGF stimulation. Because PDGF-R and integrin signaling can lead to similar changes (25), cells were plated on poly(L-lysine)-coated chamber slides, a neutral adhesive substrate. Peripheral membrane ruffles and loss of stress fibers were evident at 5 min, followed by lamellipodium formation at 10–30 min. PI3K signaling pathway at the leading edge is implicated in the early events; however, the role of individual isoforms is not known (26). Consistent with previous observations, the panPI3K inhibitor (GDC-0941) prevented PDGF-induced F-actin remodeling, including maintenance of the stress fibers and decreased formation of membrane protrusions (B. Bartok, manuscript in preparation). PI3Kdelta (INK007 or CAL-101) and PI3Kδ/γ (IPI-145) inhibitors decreased the percentage of cells with lamellipodia (Fig. 5B) in conjunction with early reappearance of stress fibers (Fig. 5A, Supplemental Fig. 1, white arrows). Thus, inhibiting PI3Kδ abrogates PDGF-induced lamellipodium formation in synoviocytes required for cell motility.

PI3Kδ inhibition reduces Rac1 activation in response to PDGF

PDGF stimulates autophosphorylation of tyrosine residues on its receptor within its cytoplasmic domain. These phosphorylated residues provide docking sites for downstream signaling molecules that reorganize the actin cytoskeleton (27). One essential event in this pathway is activation of the small guanosine triphosphatase (GTPase) Rac1, which promotes actin polymerization at the leading edge. Because Rac1 might be a target of PI3K signaling (23, 26), we examined inhibition of Rac1 activation by PI3K inhibitors in RA FLS following PDGF stim-

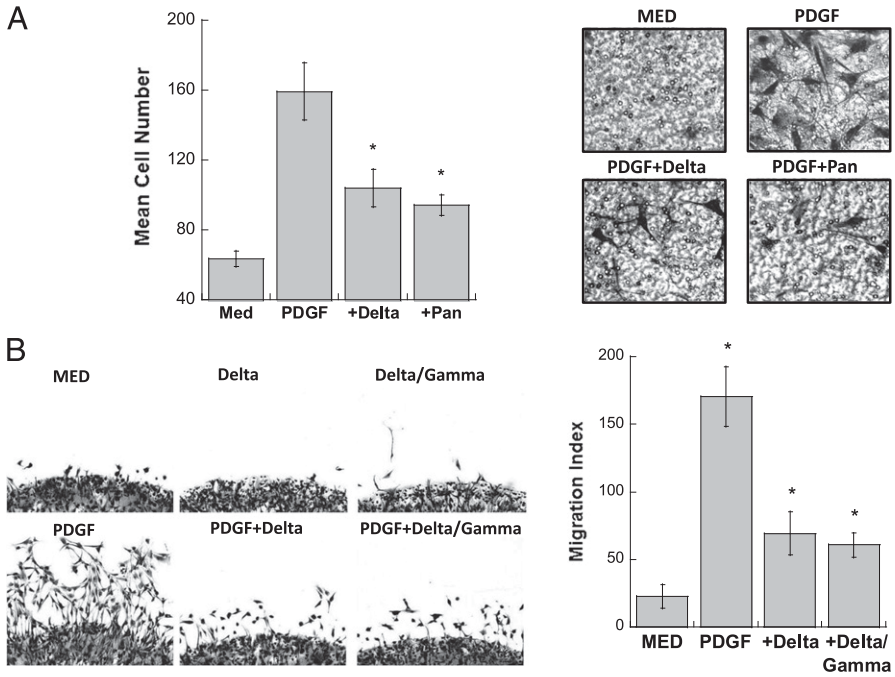

FIGURE 3. PI3Kδ inhibition decreases RA FLS migration in response to PDGF. (**A**) Chemotaxis was evaluated using a Boyden chamber migration assay toward serum-free media or supplemented with PDGF-BB (10 ng/ml). RA FLS in serum-free condition in presence of PI3Kδ INK007 (1 μM) or panPI3K GDC-0941 (0.3 μM) inhibitors or DMSO were placed in the upper chambers. Cells were allowed to migrate for 6 h, fixed, and stained with Hemacolor staining kit. The numbers of migrating cells were averaged from three ×10 field-of view images. Original magnification ×100. Data are presented as mean ± SEM of three independent experiments (*$p < 0.05$). Light microscopy images are representative of migration through the membrane after staining. (**B**) PI3Kδ inhibition decreased RA FLS migration in spot spheroid assay. RA FLS after overnight serum starvation were trypsinized and resuspended in DMEM at 2.5×10^4 cells/μl and mixed 1:1 with growth factor–reduced Matrigel (4 mg/ml). A quantity amounting to 4 μl of this mixture was spotted in a 24-well tissue culture dish and incubated at 37°C for 10 min to gel. MED (0.1% FCS) alone or with PDGF-BB (10 ng/ml) was added in the presence or absence of 1 μM INK007 (PI3Kδ) or 0.3 μM IPI-145 (PI3Kδ/γ) or DMSO. After 48 h, the organ cultures were fixed and stained with Hemacolor staining kit. Representative light microscopy images are shown that depict ~1/4 of the spot after 48 h. Original magnification ×40. Number of migrated cells was quantified using NIH ImageJ. Data are presented as mean ± SEM (*$p < 0.006$, $n = 4$ RA lines).

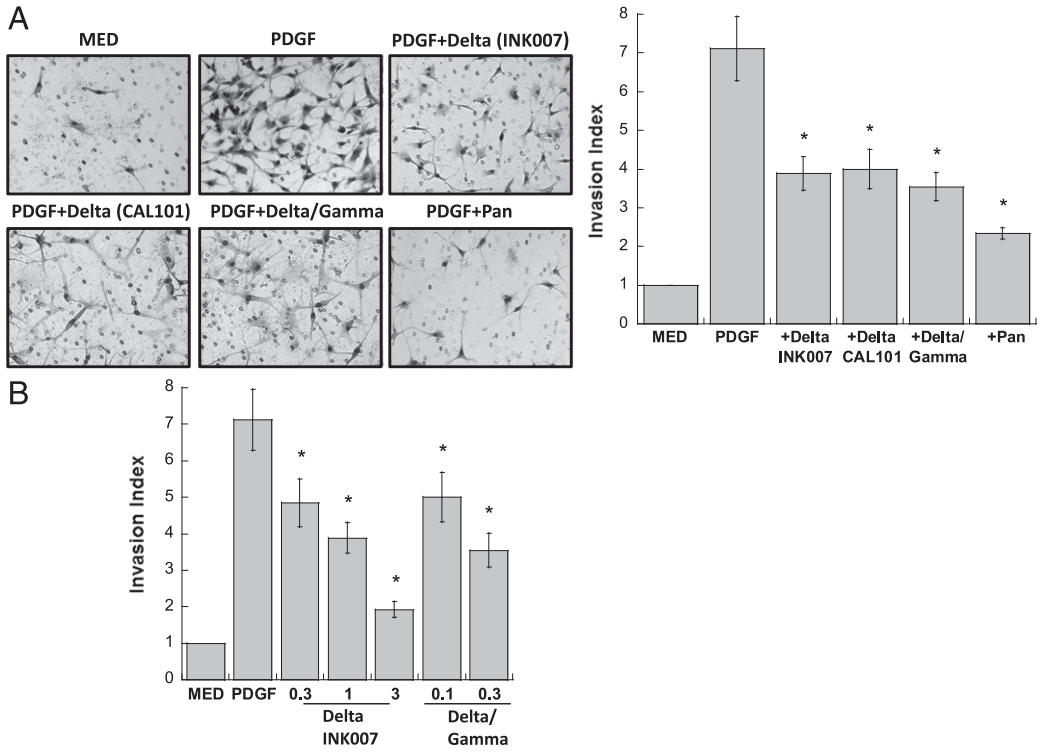

FIGURE 4. Effect of PI3K inhibition on RA FLS invasion. (**A**) Invasive migration of RA FLS was analyzed through growth factor–reduced Matrigel-coated transwell inserts in the presence or absence of 1 μM INK007 (PI3Kδ), 5 μM CAL-101 (PI3Kδ), or 0.3 μM IPI-145 (PI3Kδ/γ), or 0.3 μM GDC-0941 (panPI3K) inhibitors or DMSO. Cells were allowed to invade through Matrigel toward PDGF-BB (25 ng/ml) containing media for 24 h and were fixed and stained with Hemacolor staining kit. The number of invading cells was averaged from three ×10 field-of view images, and invasion index was calculated by normalizing the mean of invaded cells to media control. Representative images of stained cells that invaded through Matrigel invasion chambers. Original magnification ×100. Data are presented as mean ± SEM of three independent experiments (*$p < 0.04$). (**B**) Dose response of the inhibitory effect of INK007 and IPI-145 on FLS invasion.

ulation (Fig. 6A). There was profound suppression of Rac1 activation in the presence of PI3Kδ (INK007 or CAL-101) or PI3Kδ/γ (IPI-145) inhibitors compared with vehicle control (Fig. 6B, 6C).

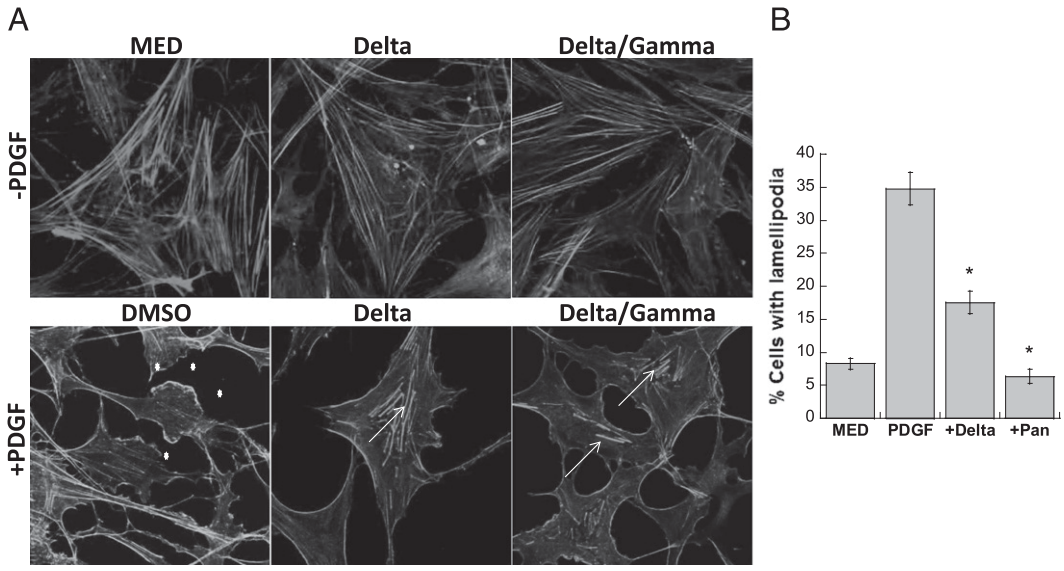

FIGURE 5. Effect of PI3Kδ inhibition on actin cytoskeleton remodeling after PDGF stimulation. (**A**) The distribution of F-actin in RA FLS was examined by immunofluorescence microscopy. Cells were cultured in poly(L-lysine)-coated chamber slides and, following 24 h of serum starvation, were stimulated with PDGF-BB (10 ng/ml) for 30 min. To determine the effect of PI3Kδ inhibition, cells were treated for 1 h prior to PDGF stimulation with the following inhibitors: 1 μM INK007 (PI3Kδ) or 0.3 μM IPI-145 (PI3Kδ/γ) or DMSO. To visualize actin remodeling, cells were fixed and stained with rhodamin-phalloidin. Representative confocal microscopy images of three independent experiments are shown to illustrate loss of stress fibers and appearance of lamellipodia in response to PGDF. Original magnification ×400. In the presence of PI3Kδ inhibitors, lamellipodia formation was suppressed and stress fibers appear (white arrow). Lamellipodia is highlighted with white asterisks. (**B**) PI3Kδ inhibitory effect on PDGF-induced lamellipodia formation. Cells were imaged with ×40 objective, and three randomly selected fields, usually containing at least 50 cells in total, were analyzed for presence of lamellipodia for each treatment condition. Data are presented as mean ± SEM of three independent experiments (*$p < 0.04$).

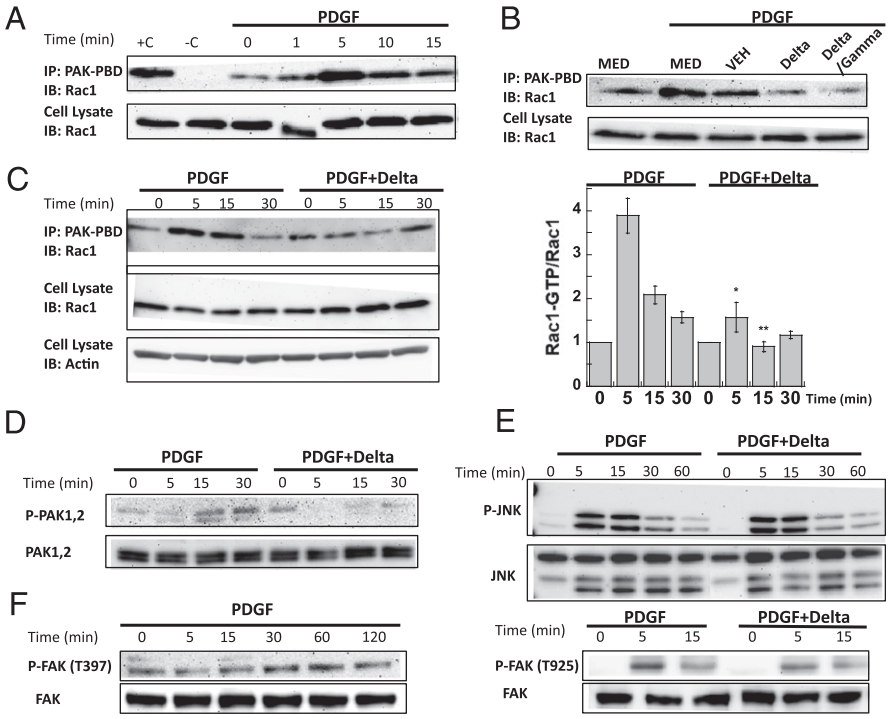

FIGURE 6. PI3Kδ inhibition decreases PDGF-mediated Rac and PAK activation in RA FLS. (**A**) PDGF induced Rac activation. FLS were serum starved for 48 h and stimulated with PDGF-BB for the indicated times. Cells were lysed, and Rac activity was determined by affinity for GST-PAK1-PBD–conjugated beads. After washing, samples were analyzed by Western blotting with anti-Rac1 Ab and were compared with total Rac activity. (**B**) Effect of PI3Kδ inhibition on Rac1 activity. Treatment with 1 μM INK007 (PI3Kδ) or 0.3 μM IPI-145 (PI3Kδ/γ) for 1 h decreased Rac1 GTPase activity at 5 min. (**C**) The effect of 1 μM INK007 (PI3Kδ) was further analyzed in a time course experiment. A representative blot of independent experiments is shown (n = 3 RA FLS lines). To quantify inhibitory effect of INK007 (PI3Kδ), Western blot densitometry was performed for Rac1-GTP and total Rac1 too. Data are shown as the ratio of arbitrary absorption units of Rac1-GTP and total Rac1 normalized to MED alone sample (mean ± SEM, $*p < 0.02$, $**p < 0.03$, $n = 3$ RA FLS lines). Effect of PI3Kδ inhibition on PDGF-induced PAK1,2 (**D**) and JNK (**E**) phosphorylation. Western blot analysis for P-PAK1,2, total-PAK, P-JNK, and total JNK following PDGF stimulation is shown in presence and absence of 1 μM INK007 (PI3Kδ). A representative blot of independent experiments is shown (n = 3 RA FLS lines). (**F**) PDGF induced phosphorylation of FAK. FLS were serum starved for 24 h and stimulated with PDGF-BB (10 ng/ml) for the indicated times. To test effect of PI3Kδ inhibition, cells were preincubated with 1 μM INK007 for 1 h prior to PDGF stimulation. Phosphorylation of FAK was analyzed by Western blot analysis using P-FAK (T397), P-FAK (T925), and FAK Abs. A representative blot of independent experiment is shown (n = 2 RA FLS lines).

Downstream activation of PAK and JNK was then analyzed, because both are also involved in cell migration (22, 28). PI3Kδ inhibition decreased PAK phosphorylation (Fig. 6D) but had no effect on P-JNK (Fig. 6E). Next, we examined whether PI3Kδ-induced Rac1/PAK1,2 activation requires FAK phosphorylation. FAK is a signaling protein that associates with integrins and growth factor receptors (29). FAK phosphorylation at T397 or T927 in response to PDGF was unchanged in presence of PI3Kδ inhibitor INK007 (Fig. 6F, data for P-FAK [T397] is not shown). These observations suggest that PI3Kδ inhibition modulates PDGF-induced cytoskeletal changes and cell migration via Rac1-PAK signaling pathway.

Discussion

The synovial lining undergoes dramatic changes in RA, leading to formation of hyperplastic, invasive tissue that invades and destroys joint structures. These hallmark pathogenic alterations can be partially ascribed to direct and indirect effects mediated by synovial fibroblasts (1, 7, 30). In addition, synoviocytes potentially promote disease progression by migrating from affected to unaffected joints. Studies using synoviocyte–cartilage cocultures in vitro or implanted in SCID mice demonstrated that RA synovial fibroblasts, but not normal or OA, exhibit cartilage invasiveness and a promigratory phenotype (3). Arthritis models using genetically modified mice that lack cadherin-11, a molecule predominantly expressed by synovial fibroblasts, confirm that FLS are primary effectors of cartilage erosion (31).

Despite the potential importance of synoviocyte-mediated joint destruction in RA, there are no therapies that directly target synoviocyte-mediated joint damage. Understanding the precise mechanism and function of the signaling pathways that regulate RA FLS migration and invasion could lead to effective novel therapies. One pathway that might participate in this process is PI3K. This signaling mechanism plays a pivotal role in regulating cell migration in some cell types, and altered activation can lead to pathogenic migration and invasion (32–34).

The class I PI3Ks (PI3Kα, β, γ, and δ) are a family of lipid kinases that are structurally related, forming heterodimers between a catalytic subunit and one of the several regulatory subunits. They mediate their biological activity via generation of the secondary messenger phosphatidylinositol-(3–5)-triphosphate at the cell membrane, which interacts with several effector proteins, including Akt (6). PI3Ks regulate diverse cellular functions, including proliferation, survival, and migration. Various PI3K isoforms have distinct functions during cell migration that are cell type and stimulus dependent (6, 26).

Among the four class I PI3K isoforms, PI3Kδ might be especially relevant in RA. Not only is it highly expressed in RA synovial lining compared with OA, but also PI3Kδ is expressed by cultured FLS and induced by inflammatory cytokines (4, 35). We

previously showed that PI3Kδ in synoviocytes is engaged downstream of the PDGF-R and regulates growth and survival via Akt activation (12). PI3Kδ is also important for leukocyte chemotaxis in physiological and pathologic responses such as inflammation and hematologic malignancy (8, 10, 36, 37). PI3Kδ activity has been linked to cancer cell migration in breast cancer, neuroblastoma, and glioblastoma, and, aberrant expression of PI3Kδ in tumors might contribute to the malignant properties of cancer cells (37–39). The role of PI3K in tumor cells could be directly relevant to the aggressive nature of RA FLS.

The importance of PI3K signaling in cancer is highlighted by the fact that gain-of-function mutations in PIK3CA, the gene encoding PI3Kα, are present in a wide variety of human solid tumors (40–43). In contrast, somatic mutations of the genes encoding PI3Kδ have not been observed (37). However, overexpression of wild-type PI3Kδ induces oncogenic characteristics in chicken embryo fibroblasts and has been noted in several tumor types (44). Its expression is complex and is controlled by multiple distinct promoters. The TNF-inducible promoter is active in synoviocytes but not in leukocytes (35). Based on these observations, we considered whether PI3Kδ might also regulate RA FLS migration and invasion.

Recent development of small-molecule PI3K inhibitors with increased selectivity provides tools to test our hypothesis (8, 11). In the current study, we used multiple compounds with distinct structures as well as genetic approaches to confirm that the effects are due to PI3Kδ inhibition rather than off-target effects. Diminished baseline and stimulated migration of RA FLS in the presence of panPI3K inhibitors suggests that FLS migration is PI3K dependent and was confirmed by siRNA knockdown experiments. PI3Kδ inhibition had no effect on baseline migration but significantly decreased migration in response to PDGF in the wound-healing and chemotaxis assays. Similar results were obtained when FLS were embedded in Matrigel to mimic the in vivo three-dimensional environment.

Directional cell migration is a tightly coordinated process that is initiated by cell adhesion, polarization, and actin cytoskeleton rearrangement. This, in turn, leads to membrane protrusions that extend to form a lamellum at the leading edge to dictate the direction of movement. Subsequently, localized actin polymerization at the lamellipodium is required for generating propulsive force that mediates forward movement (22, 31, 45). To gain insight into mechanisms by which PI3Kδ regulates migration, we examined actin cytoskeleton remodeling associated with cell migration. Alteration of actin polymerization, decreased lamellipodium formation, and early reappearance of stress fibers with PI3Kδ inhibition indicate that PI3Kδ activity is required for protrusion formation and migration during PDGF stimulation. This is consistent with the phenotype observed in breast cancer cells microinjected with anti-p110δ Ab (39).

The Rho GTPases serve as molecular switches and are central regulators of actin cytoskeleton dynamics during directional migration. Rac1 drives actin polymerization to promote lamellipodium formation required for efficient migration (22, 31, 45). Our studies showed that PI3Kδ inhibition decreased Rac1 activity as well as its downstream effector PAK1 in FLS. The results suggest that Rac1 is a key target of PDGF-mediated PI3Kδ signaling. PI3Kδ inhibition had no effect on P-FAK or P-JNK, indicating that these pathways are not involved. FAK is among the first proteins activated by integrin signaling and mediates Rac1 activation following integrin–extracellular matrix interaction. Our migration assay was independent of integrins, which is probably why PI3Kδ inhibition had no effect on P-FAK.

PI3K-generated phospholipids serve as second messenger that directly activates Rac or Rac-GEFs. Several Rac GEFs are activated by phosphatidylinositol-(3–5)-triphosphate, including Vav, Tiam1, PIX, and p-REX (46). Rac activity may be sustained through feedback loops, as Rac can interact with the p85 regulatory subunit of class 1A PI3Ks in leukocytes, although possibly not in fibroblasts (26, 47). Although activation of Akt downstream of PI3K is implicated in cell migration via Rac/Pak pathway, the mechanism of Akt activation is poorly understood (48–51). Thus, PI3K-mediated Rac activation can occur in Akt-dependent and/or independent manner during cell migration. However, further studies are needed to dissect the precise mechanism of Rac1 regulation by PI3Kδ in synoviocytes.

Another important observation of the current study is that PI3Kδ is required for effective FLS invasion into the extracellular matrix, thereby describing a new function of PI3Kδ. This characteristic could have clinical implications, because in vitro invasiveness of RA FLS is associated with joint destruction in RA (52). A therapeutic agent that decreases movement of FLS between joints as well as pathogenic invasion into cartilage could decrease tissue damage and loss of function.

In conclusion, our findings, together with previous studies showing that PI3Kδ regulates FLS growth and survival as well as TNF and IL-1 signaling, suggest that PI3Kδ is an attractive therapeutic target for RA. Blocking this isoform would potentially suppress innate and adaptive immune responses, especially related to B cell activation, that play a pivotal role in RA. The beneficial effect on FLS migration and invasion through a distinct mechanism offers the potential for enhanced joint protection.

Disclosures

The authors have no financial conflicts of interest.

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