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TNF α , PDGF and TGF β synergistically induce synovial lining hyperplasia via inducible PI3K δ --Manuscript Draft--

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Corresponding Author:	Hiroyuki Yoshitomi JAPAN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Hideyuki Shibuya
First Author Secondary Information:	
Order of Authors:	Hideyuki Shibuya Hiroyuki Yoshitomi Koichi Murata Shio Kobayashi Moritoshi Furu Masahiro Ishikawa Takayuki Fujii Hiromu Ito Shuichi Matsuda
Order of Authors Secondary Information:	
Abstract:	Objectives To determine the mechanism underlying hypertrophic synovium in rheumatoid arthritis (RA). Methods We examined micromass cultures of fibroblast-like synoviocytes (FLSs) stimulated with tumor necrosis factor α (TNFα), platelet-derived growth factor (PDGF), and/or transforming growth factor β (TGFβ). The hypertrophic architecture of the micromasses, expression of phosphoinositide 3 kinase (PI3K) isoforms, and persistent activation of PI3K-Akt pathways were investigated. FLSs transfected with siRNA were also examined in the micromass cultures. Results The combination of TNFα, PDGF and TGFβ (TPT condition) induced obvious hypertrophic architecture of the intimal lining layer in FLSs in micromass cultures, and was accompanied by upregulated expression of matrix metalloproteinase-3 (MMP3), Cadherin-11, and PI3Kδ. In monolayer FLSs, the TPT condition enhanced the expression of PI3Kδ and persistent activation of the PI3K-Akt pathway. Knockdown of PI3Kδ significantly inhibited the formation of the hypertrophic synovial lining in the TPT condition. Conclusions These results collectively indicate that inducible PI3Kδ plays a crucial role in persistent activation of PI3K-Akt in FLSs, and in the formation of a hypertrophic synovial lining. PI3Kδ may be an alternative treatment target for the regulation of proliferative synovium in RA.

Suggested Reviewers:	Katsuaki Kanbe kanbeor@dnh.twmu.ac.jp
	Takaki Nojima nojima@kuhp.kyoto-u.ac.jp
	Norifumi Sawamukai sawa@med.uoeh-u.ac.jp
Opposed Reviewers:	

**TNF α , PDGF and TGF β synergistically induce synovial lining hyperplasia via
inducible PI3K δ**

Hideyuki Shibuya¹, M.D., Hiroyuki Yoshitomi^{1,2}, M.D.,PhD., Koichi Murata¹, M.D.,PhD., Shio
Kobayashi², Moritoshi Furu^{1, 3}, M.D.,PhD., Masahiro Ishikawa¹,M.D.,PhD., Takayuki Fujii¹, M.D.,
Hiromu Ito^{1,3},M.D.,PhD. and Shuichi Matsuda¹M.D.,PhD.

¹Department of Orthopaedic Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan
²Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University
Graduate School of Medicine, Kyoto, Japan
³Department of the Control for Rheumatic Diseases, Kyoto University Graduate School of Medicine,
Kyoto, Japan

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Address correspondence and reprint request to:
Hiroyuki Yoshitomi
Center for Innovation in Immunoregulative Technology and Therapeutics, Kyoto University
Graduate School of Medicine
Address: Yoshidakonoe-cho, Sakyo, Kyoto 606-8501, Japan
TEL +81-75-753-9501
FAX +81-75-753-9500
E-mail: yositomi@kuhp.kyoto-u.ac.jp

ABSTRACT

Objectives To determine the mechanism underlying hypertrophic synovium in rheumatoid arthritis (RA).

Methods We examined micromass cultures of fibroblast-like synoviocytes (FLSs) stimulated with tumor necrosis factor α (TNF α), platelet-derived growth factor (PDGF), and/or transforming growth factor β (TGF β). The hypertrophic architecture of the micromasses, expression of phosphoinositide 3 kinase (PI3K) isoforms, and persistent activation of PI3K-Akt pathways were investigated. FLSs transfected with siRNA were also examined in the micromass cultures.

Results The combination of TNF α , PDGF and TGF β (TPT condition) induced obvious hypertrophic architecture of the intimal lining layer in FLSs in micromass cultures, and was accompanied by upregulated expression of matrix metalloproteinase-3 (MMP3), Cadherin-11, and PI3K δ . In monolayer FLSs, the TPT condition enhanced the expression of PI3K δ and persistent activation of the PI3K-Akt pathway. Knockdown of PI3K δ significantly inhibited the formation of the hypertrophic synovial lining in the TPT condition.

Conclusions These results collectively indicate that inducible PI3K δ plays a crucial role in persistent activation of PI3K-Akt in FLSs, and in the formation of a hypertrophic synovial lining. PI3K δ may be an alternative treatment target for the regulation of proliferative synovium in RA.

Keywords:

Fibroblast-like synoviocytes

PI3K/Akt pathway

PI3K δ

PDGF

TGF β

Introduction

Synovial hyperplasia refers to rheumatoid arthritis (RA) lesions responsible for cartilage degeneration, osteoclast induction, and subsequent joint deformities [1].

Clinically, the swollen joint count of patients with RA is associated with the progression of joint damage in RA more than is systematic inflammation [2]. Although there are various types of cells in RA synovium and joints, including fibroblast-like synoviocytes (FLSs), macrophages, T cells, B cells, dendritic cells, neutrophils, and osteoclasts, and while each is considered crucial for RA pathogenesis, FLSs play a key role in synovial hyperplasia by constructing the main framework of RA synovium [1,3,4,5].

Platelet-derived growth factor (PDGF) and transforming growth factor β (TGF β) are abundant in the synovium and synovial fluid in RA [6,7,8]. PDGF is considered to be involved in the pathogenesis of RA as a growth factor of FLSs [7,8]. On the other hand, TGF β is more complicated; it has an inhibitory effect in lymphocytes and macrophages [9], but induces proliferation and aggrecanase production in FLSs [10,11]. The combination of PDGF and TGF β synergistically activates extracellular signal-regulated kinase and phosphoinositide 3 kinase (PI3K) pathways of FLSs and

enhances the production of inflammatory mediators in monolayer FLSs [12].

Monolayer culture or invasion assays of FLSs are frequently used but have difficulty in the analysis of factors relating to hypertrophic architecture formed by FLSs. Kiener et al. showed that a micromass culture of human FLSs formed lining/sublining architecture resembling synovial tissues, and found that $\text{TNF}\alpha$ and PDGF contributed to the hypertrophic architecture of intimal lining layer to some extent [13]. However, further mechanisms underlying the hypertrophy of intimal lining driven by FLSs remain to be determined.

In this study, we show that the combination of $\text{TNF}\alpha$, PDGF, and $\text{TGF}\beta$ synergistically induces obvious hypertrophic architecture in FLS micromass culture, and that inducible PI3K δ plays a key role in the persistent activation of the PI3K-Akt pathway, and in the formation of hypertrophic FLS architecture.

Materials and Methods

Isolation of fibroblast-like synoviocytes (FLSs)

Ethical approval for using human samples for this study was granted by the Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. Written informed consent was obtained from all study participants. RA was diagnosed in accordance with the criteria of the American College of Rheumatology [14]. FLSs from patients with RA were prepared and cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with penicillin (Sigma), streptomycin and 10% FBS (Sigma), as previously described [15].

Micromass culture of FLSs

1
2 Micromass cultures of FLSs were prepared, using a process previously reported
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4 [13]. Briefly, FLSs were suspended in ice-cold Matrigel Matrix (BD Biosciences,
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6 Bedford, MA) at a density of 5×10^6 cells/ml. Droplets (40 μ l) of the suspension were
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8 placed onto poly-2-hydroxyethylmethacrylate (poly-HEMA; Sigma)-coated culture
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10 dishes. Gelation was allowed to occur for 45 min at 37°C. The gel was then overlaid
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12 with culture medium. The floating 3-D culture was maintained for 2–3 weeks with a
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14 medium change twice a week.
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19 For stimulation experiments, FLS micromasses were cultured in basal medium
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21 containing 10 ng/ml of tumor necrosis factor α (TNF α), 10 ng/ml of platelet-derived
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23 growth factor (PDGF), and/or 10 ng/ml of transforming growth factor β (TGF β) (each
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25 from Pepro Tech; Rocky Hill, NJ).
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32 *Histological analysis*

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34 Micromasses were processed as 20- μ m-thick frozen sections and stained with
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36 hematoxylin and eosin. Light microscopic images were taken using ECLIPSE 80i
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38 (Nikon, Japan) with a 10 $\times$ dry lens. The area of the intimal lining layer, including
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40 villous structures intruding toward the center of the micromass, was measured using
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42 ImageJ (NIH).
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49 Quantitative real-time PCR (qRT-PCR)

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51 Reverse transcription was performed using a Transcriptor Universal cDNA Master
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53 (Roche Applied Science, Indianapolis, IN). qRT-PCR was performed using FastStart
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55 Universal SYBR Green Master (Roche Applied Science) on an Applied Biosystems
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7500 Thermocycler according to the manufacturer's protocol. The following primer sequences were used: *GAPDH*: forward, 5'-TCTCGCTCCTGGAAGATGGT-3'; reverse, 5'-GGAAGGTGAAGGTCGGAGTC-3'; *MMP3*: forward, 5'-GATACCCAAGAGGCATCCAC-3'; reverse, 5'-CTGGCTCCATGGAATTTCTC-3'; *Cadherin11*: forward, 5'-AGAGAGCCCAGTACACGTTGA-3'; reverse, 5'-TTGGCATGATAGGTCTCGTGC-3'; *PI3K α* : forward, 5'-CCACGACCATCATCAGGTGAA-3'; reverse, 5'-CCTCACGGAGGCATTCTAAAGT-3'; *PI3K β* : forward, 5'-AGAGCACTTGGTAATCGGAGG-3'; reverse, 5'-CTTCCCCGGCAGTATGCTTC-3'; *PI3K γ* : forward, 5'-GGCGAAACGCCCATCAAAAA-3'; reverse, 5'-GACTCCCGTGCAGTCATCC-3'; and *PI3K δ* : forward, 5'-AAGGAGGAGAATCAGAGCGTT-3'; and reverse, 5'-GAAGAGCGGCTCATACTGGG-3'. The expression of each mRNA was normalized by the expression of GAPDH.

Western blot analysis

Western blot analysis was performed as previously described [3]. Briefly, 10–20 ng of cell lysate was subjected to 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blocking with 1% skim milk or 2% BSA, the membrane was probed with rabbit anti- β -tubulin (clone 11H10), rabbit anti-Akt (clone 11E7), or rabbit anti-phospho-Akt (Ser473: clone D9E) (all purchased from Cell Signaling Technology; Beverly, MA), incubated with horseradish

protein-conjugated goat anti-rabbit IgG (Cayman Chemical, Ann Arbor, MI), and visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL), ECL Plus Western Blotting Detection Reagent (GE Healthcare UK, Buckinghamshire, UK), or ECL Prime Western Blotting Detection Reagent (GE Healthcare UK), as appropriate.

Cell-based enzyme-linked immunosorbent assay (ELISA)

Cell ELISA was performed as previously described [3]. Briefly, FLSs were cultured for 24 h, stimulated with cytokines, fixed with 4% paraformaldehyde, permeabilized with TBS containing 0.001% Triton X, quenched with PBS containing 0.1 M glycine and 1% H₂O₂, and blocked with TBS with 2% BSA solution, incubated with primary antibody as for Western blottings, incubated with horseradish protein-conjugated goat anti-rabbit IgG, and reacted with a substrate solution containing 3,3',5,5'-tetramethylbenzidine (Sigma). The reaction was stopped with 2 N sulfuric acid. The optical density was measured at 450–630 nm with a microplate reader. Linearity of ELISA was confirmed using serially diluted samples.

siRNA

FLSs were transfected with double-stranded siRNA (Stealth RNAi; Life Technologies) for 48 h using a TransIT-TKO transfection reagent (Mirus) as per the manufacturer's protocol. The siRNA sequences are as follows; siCTL: sense, 5'-AAGUGA AUGUGCGUUUGGAACGUGC-3'; antisense, 5'-GCACGUUCCAAACGCACAUUCACUU-3'; siPI3K δ ,

5'-GGGCCAACCTCATGCTGTTTGACTA-3'; and antisense,

5'-TAGTVAAAVAGCATGAGGTTGGCCC-3'.

Results

The combination of $\text{TNF}\alpha$, PDGF, and $\text{TGF}\beta$ induces hypertrophy of the intimal lining layer in micromass cultures of FLSs.

Hypertrophy of synovial tissue, especially in the lining layer, is characteristic of lesions observed in RA patients. To determine whether the combination of PDGF and $\text{TGF}\beta$ has a synergistic effect in the formation of hypertrophic synovium, we conducted micromass culture of human FLSs under stimulation with $\text{TNF}\alpha$, PDGF, and/or $\text{TGF}\beta$. Micromass cultures in a normal medium showed lining/sublining architecture, as previously reported (Fig. 1A). The individual use of $\text{TNF}\alpha$, PDGF and $\text{TGF}\beta$ in the culture medium at a concentration of 10 ng/ml slightly thickened the intimal lining layer (Fig. 1B, C and D), and higher concentrations (100 ng/ml) of each cytokine had a similar effect as when stimulating at a concentration of 10 ng/ml (data not shown). Interestingly, PDGF or $\text{TGF}\beta$ frequently showed villous architecture of the intimal lining intruding toward the center of FLS micromass cultures (Fig. 1C and D). The addition of $\text{TNF}\alpha$ to PDGF or $\text{TGF}\beta$ did not enhance the hypertrophy effect of PDGF or $\text{TGF}\beta$, whereas the combination of $\text{TNF}\alpha$, PDGF, and $\text{TGF}\beta$ (TPT condition) significantly increased the thickness of the intimal lining and villus formation (Fig. 1H and I). Thus, the combination of $\text{TNF}\alpha$, PDGF, and $\text{TGF}\beta$, each present abundantly in the synovial fluid, induced the hyperplastic architecture of FLSs.

PI3K δ was upregulated in FLS micromasses cultured in the TPT condition.

In the hyperplastic synovium of RA, various molecules are elevated and contribute to the pathogenesis of RA. Metalloproteinases, including MMP3, contribute to cartilage erosion and tendon rupture by degenerating the collagen matrix [17-19]. Cadherin-11 plays a crucial role in synovial structure by supporting cell-to-cell junctions. In the micromass organ culture, the expression of MMP3 was apparently correlated with the addition of TNF α (Fig. 2A), whereas Cadherin-11 was expressed most in the TPT condition (Fig. 2B). The elevation of PI3K δ is also reported in RA synovium [19]. Among the isoforms of PI3K, the expression of PI3K δ significantly increased in micromass culture under the TPT condition (Fig. 2C-E), whereas PI3K γ was undetectable in almost all samples (data not shown). Thus, micromass culture of FLSs accompanying hypertrophic architecture showed the upregulation of molecules characteristic of RA hyperplastic synovial tissues.

TPT condition enhanced the expression of PI3K δ and persistently activated the PI3K-Akt pathway in monolayer FLSs.

To further determine the signal pathway relating to the TPT condition, we stimulated monolayer FLSs with the TPT condition, and confirmed the expression of PI3K isoforms and the activation of the PI3K-Akt pathway. Stimulation of monolayer FLSs showed an expression pattern of PI3K isoforms similar to that of micromass cultures of FLSs; the expression of PI3K δ was significantly increased by the stimulation of the TPT condition (Fig. 3A). On the other hand, stimulation of monolayer FLSs with the TPT condition failed to enhance the expression of Cadherin-11, implying the

involvement of the 3-D architecture of the intimal lining in the expression of Cadherin-11 (data not shown). As previously reported, TNF α did not enhance the phosphorylation of Akt, whereas the other conditions, including PDGF alone, the combination of TNF α and PDGF, and the TPT condition, each similarly increased phosphorylated Akt (P-Akt) 6 h after the stimulation (Fig. 3B). The TPT condition also increased the amount of P-Akt 24 h after the stimulation compared with other conditions (Fig. 3B). The time course of P-Akt, measured by cell-ELISA, showed that its increase under stimulation with PDGF was similar to that under stimulation with TNF α and PDGF together, and with the TPT condition for the first 6 h. In the subsequent 18 h, the amount of P-Akt under the condition of PDGF, and with the combination of PDGF and TNF α , gradually decreased, but continued to increase in the TPT condition (Fig. 3C and D). The amount of P-Akt in the TPT condition was significantly higher than that of other conditions at both 24 h and 48 h after stimulation (Fig. 3E and F). These data indicate that stimulation of monolayer FLSs with the TPT condition enhances PI3K δ and persistently activates the PI3K-Akt pathway.

Knockdown of PI3K δ in monolayer FLSs attenuated the activation of the PI3K-Akt pathway induced by the TPT condition.

To determine the involvement of PI3K δ in the activation of the PI3K-Akt pathway, we knocked down the expression of PI3K δ . First, we confirmed that transfection of siPI3K to monolayer FLSs specifically downregulated the mRNA of PI3K δ but not that of PI3K α , nor PI3K β (Fig. 4A). PI3K δ siRNA significantly attenuated the phosphorylation of Akt induced by the TPT condition at each time point

1 during 48 h (Fig. 4B). These results indicate that inducible PI3K δ of FLSs plays a
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3 crucial role in the persistent activation of the PI3K-Akt pathway induced by the TPT
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6 condition.
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12 PI3K δ is involved in hyperplastic synovial lining.
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15 We have shown that the TPT condition induces hypertrophy of the intimal
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17 lining layer and also enhances the expression of PI3K δ in Figures 1 and 2. To determine
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19 whether PI3K δ is functionally involved in the formation of hypertrophic synovial lining,
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21 PI3K δ -knocked down FLSs were conducted 3-D culture under the TPT condition. We
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23 confirmed that the knockdown of PI3K δ persisted for at least 10 days (data not shown).
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25
26 Knockdown of PI3K δ significantly inhibited the thickening of the intimal lining layer
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28 induced by the TPT condition (Fig. 4C and D). Thus, these results collectively suggest
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30 that the TPT condition induces hypertrophic architecture in synovium by elevating the
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32 expression of PI3K δ , and then activating the PI3K-Akt pathways.
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40 Discussion

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42 Clinical reports showed that synovial hyperplasia of RA contributed to the
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44 deformation of joints [2]. However, there is no clear understanding of how FLSs,
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46 which consist of a main framework of synovium, are regulated to lead to hyperplastic
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48 synovium in RA. In this study, we have shown that the combination of TNF α , PDGF,
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50 and TGF β synergistically induced obvious hypertrophic architecture in FLS micromass
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52 cultures accompanying upregulation of PI3K δ expression, and that inducible PI3K δ
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55 plays a crucial role in the activation of the PI3K-Akt pathway and in the hypertrophy of
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the FLS intimal lining.

A normal synovium is an acellular structure with a thin intimal lining. In RA, a massive increase of macrophage-like synoviocytes and FLSs accompanies the infiltration of T cells and B cells to lead to hyperplastic RA synovium. Interestingly, micromass cultures of FLSs form lining/sublining architecture similar to normal synovial architecture, whereas skin fibroblasts form a solid structure. This indicates that FLSs, by their nature, play a crucial role in forming architectural structures of synovial tissue [13]. Furthermore, the addition of cytokines to micromass cultures induced hypertrophic architecture. Thus, micromass cultures of FLSs are suitable experimental systems for determining the factors affecting FLSs to induce hyperplastic architecture.

TGF β , TNF α , and PDGF are abundantly present in the RA synovial environment [20-22]. TGF β is a kind of growth factor, but unlike PDGF, it has inhibitory effects and proliferative effects depending on the target cells [9-11,23]. In monolayer FLSs, the combination of PDGF and TGF β synergistically enhances the production of MMP3, IL-8, and MIP1a induced by TNF α [12], and activates the PI3K-Akt pathway [24]. In addition, we have shown that this combination also has a synergistic effect in the thickening of the synovial lining. Long-lasting activation of the PI3K-Akt pathway (Fig. 3C) might contribute to the architectural change in micromasses of FLSs.

PI3K has constitutive isoforms of PI3K α and PI3K β , and inducible isoforms of PI3K γ and PI3K δ . A deficiency of the constitutive isoforms, PI3K α or PI3K β , is embryonic lethal [25,26], implying the difficulty of treating RA by targeting these molecules. On the other hand, PI3K δ is preferentially expressed by lymphoid cells [27]

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2 and RA hypertrophic synovium [19], and a deficiency of PI3K δ does not affect the
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4 lifespan, and ameliorates arthritis in mice [28,29]. Although B cells were considered to
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6 be the most responsible cells for this amelioration, our study showed that inducible
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8 PI3K δ of FLSs is also involved in the activation of the PI3K-Akt pathway and synovial
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10 lining hyperplasia (Fig. 4B and 4C).
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15 This study has several limitations. The influence of other cytokines, such as
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17 IL-1 β or IL-6, on synovial lining hyperplasia was not investigated. The mechanism
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19 underlying the upregulation of PI3K δ in the TPT condition also remains to be
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21 determined. However, we have shown that the TPT condition clearly contributes to an
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23 architectural change in the synovial lining layer via inducible PI3K δ , possibly by
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25 persistent activation of the PI3K-Akt pathway using FLS micromass culture. Further
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27 clarification of the mechanism by which FLSs regulate the 3-D architecture should lead
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29 to an alternative treatment for RA.
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50 **Conflict of interest** None.
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54 **Figure legends**

55 **Fig. 1.** Micromass cultures of RA FLSs with cytokine stimulation. **A–H,** The frozen
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sections of micromass were stained with hematoxylin and eosin. Human FLS micromasses were cultured for 21 days in the presence of basal medium (A), with $\text{TNF}\alpha$ (B), $\text{TGF}\beta$ (C), PDGF (D), PDGF and $\text{TNF}\alpha$ (E), $\text{TNF}\alpha$ and $\text{TGF}\beta$ (F), PDGF and $\text{TGF}\alpha$ (G), and a combination of $\text{TNF}\alpha$, PDGF and $\text{TGF}\alpha$ (TPT condition) (H). The concentration of each cytokine was 10 ng/ml. Insets indicate scaled-up images of intimal linings. Open and closed arrowheads indicate villous architecture and the border between the thickening lining layer and sublining layer, respectively. **I**, Percentages of area for intimal linings in total area of a micromass. Data are shown as mean $\pm$ SEM of sextuplet. * = $P < 0.05$ in one-way ANOVA.

Fig. 2. Expression of markers relating to RA synovium in FLS micromasses under the TPT condition. **A–E**, Expression of MMP3 (A), Cadherin-11 (B), and PI3K isoforms (C–E), in micromass culture of RA FLSs in response to $\text{TNF}\alpha$, $\text{TGF}\beta$, and PDGF. Quantitative PCR was performed to quantify mRNA levels in micromass cultures. Data are normalized to GAPDH. Expression of PI3K γ was not detectable (not shown). MMP3, Cadherin-11, and PI3K δ were expressed most in the TPT condition. PI3K α and β were not affected by stimulation. Data are shown as mean $\pm$ SEM of sextuplet. * = $P < 0.05$ in one-way ANOVA.

Fig. 3. Analysis of PI3K-Akt pathway in monolayer FLSs under the TPT condition. **A**, Expression of PI3K δ mRNA in FLSs under the indicated condition for 24 h. Data are shown as mean $\pm$ SEM of quadrants. * = $P < 0.05$ in one-way ANOVA. **B**, Expression of phospho-Akt (P-Akt), total-Akt (T-Akt), and β -tubulin protein in FLSs cultured under

indicated conditions for 6 h or 24 h was analyzed by Western blot assay. **C–E**, Expression of P-Akt and T-Akt was measured by ELISA. Relative P-Akt expression during 48 h after cytokine stimulation (C), and at 24 h (D), and 48 h (E), are shown. Data are shown as mean $\pm$ SEM of triplicate. * = $P < 0.05$ in two-way ANOVA.

Fig. 4. Analysis of PI3K-Akt pathway and formation of hyperplastic architecture in PI3K δ -knocked down FLSs. **A**, Expression of PI3K isoforms in monolayer FLSs after transfection with PI3K δ siRNA or control siRNA. RA FLSs were incubated for 24 h, then transfected for 48 h. Cells were cultured in basal medium for 4 days, then mRNA was isolated. Data are shown as mean $\pm$ SEM of sextuplet. * = $P < 0.05$ in Student's t-test. **B**, The relative expression of phospho-Akt in monolayer transfected FLSs under the TPT condition was measured by cell ELISA. Data are shown as mean $\pm$ SEM of sextuplet. * = $P < 0.05$ in two-way ANOVA. **C and D**, Micromasses of FLSs transfected with siPI3K δ or siCTL were cultured in the TPT condition for 14 days. Hematoxylin and eosin stain of micromasses (C) and the percentages of lining layer area (D) are shown. Data are shown as mean $\pm$ SEM of sextuplet. * = $P < 0.05$ in Student's t-test.

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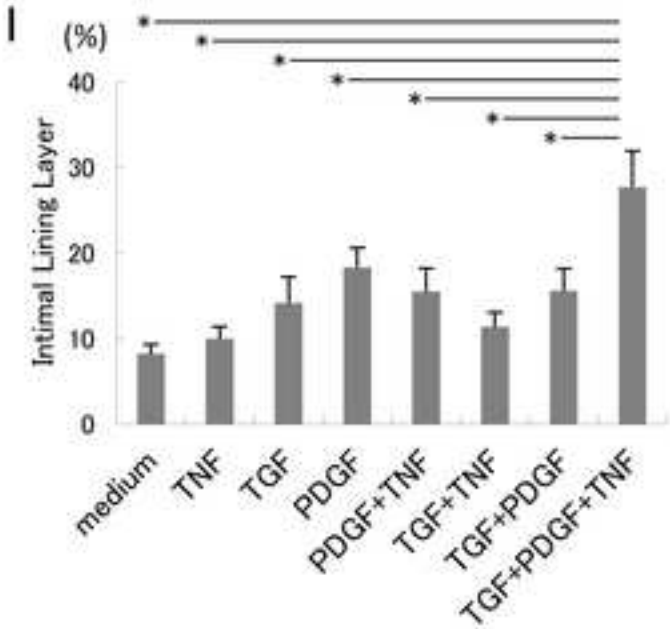
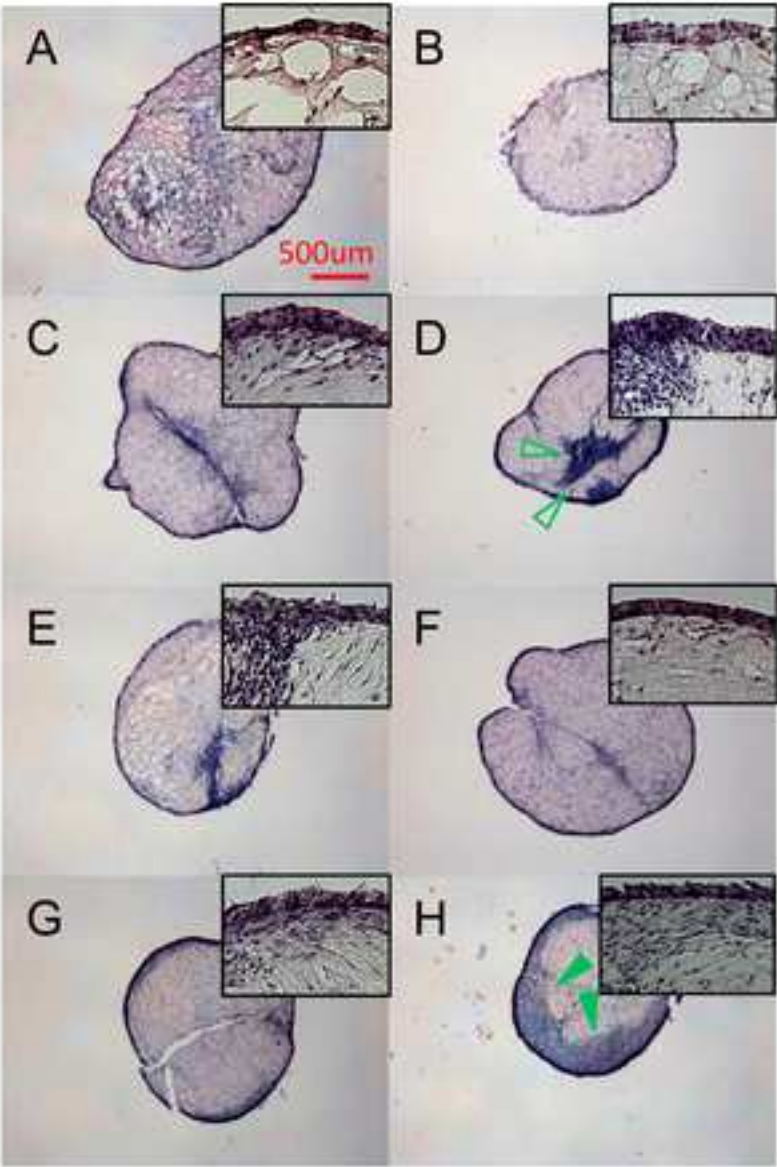


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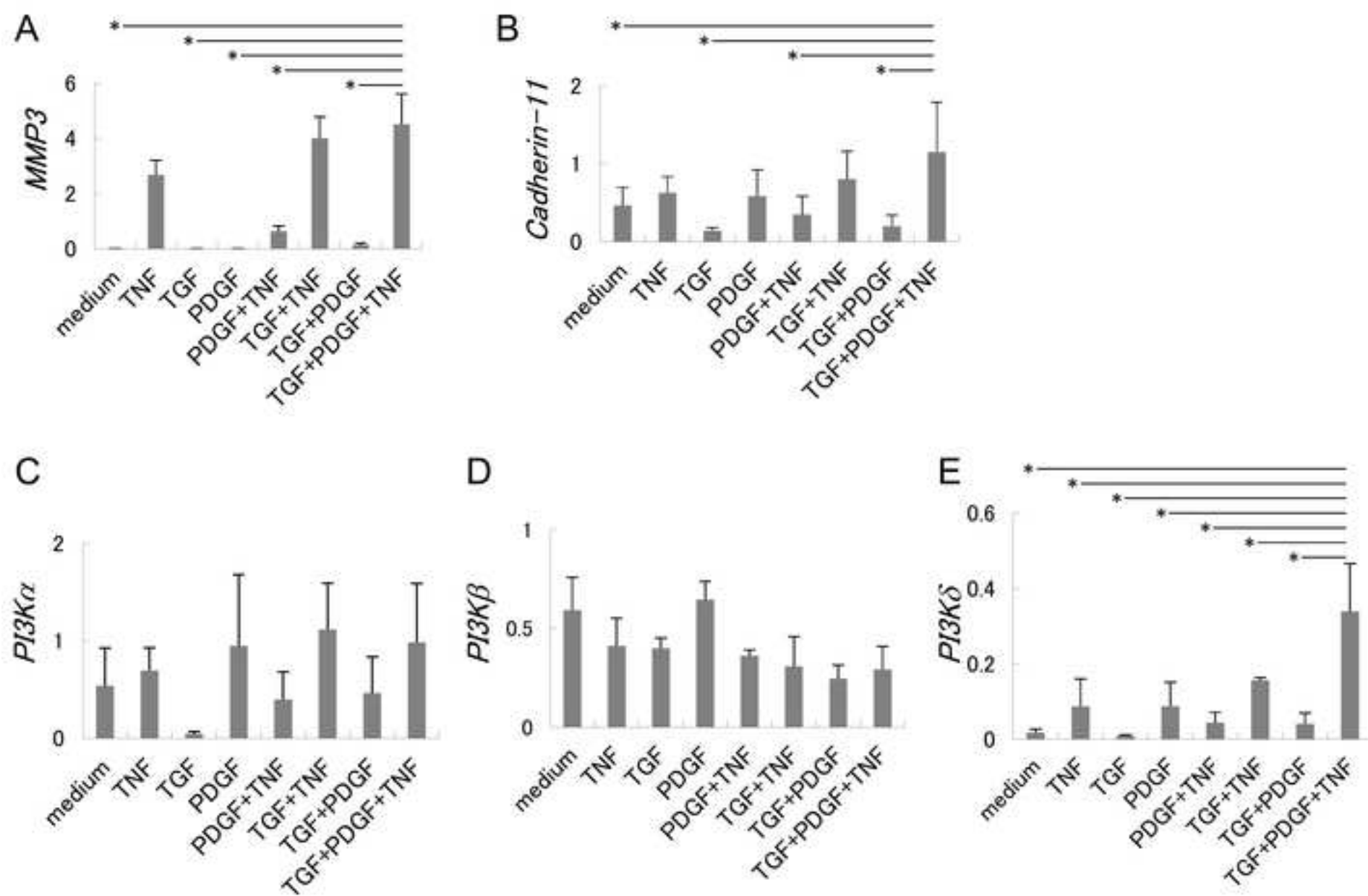


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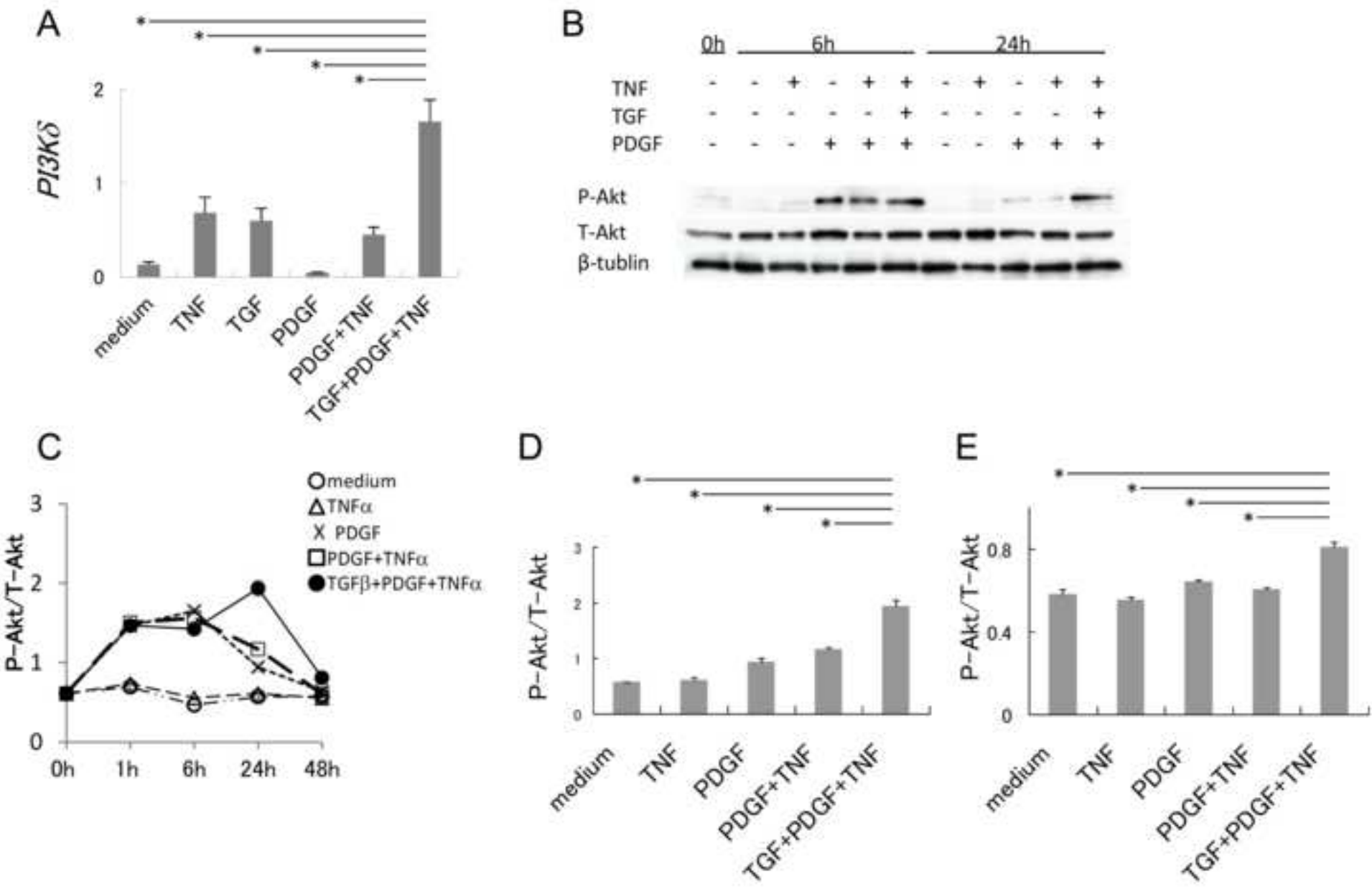


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