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Title Page

Regulation of Lysophosphatidic Acid Receptor Expression and Function in Human Synoviocytes: Implications for Rheumatoid Arthritis?

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Running Title Page

a) **Running Title:** Functional expression of LPA receptors in human FLS

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d) **Abbreviations:** LPA, lysophosphatidic acid; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; ATX, autotaxin; IL, interleukin; TNF-α, tumor necrosis factor-α, MAPK, mitogen-activated protein kinase.

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Abstract

Lysophosphatidic acid (LPA), via interaction with its G-protein coupled receptors, is involved in various pathological conditions. Extracellular LPA is mainly produced by the enzyme autotaxin (ATX). Using fibroblast-like synoviocytes (FLS) isolated from synovial tissues of patients with rheumatoid arthritis (RA), we studied the expression profile of LPA receptors, LPA-induced cell migration, as well as interleukin-8 (IL-8) and interleukin-6 (IL-6) production. We report that FLS express LPA receptors LPA_{1-3}. Moreover, exogenously applied LPA induces FLS migration and secretion of IL-8/IL-6, whereas the LPA_3 agonist OMPT stimulates cytokine synthesis but not cell motility. The LPA-induced FLS motility and cytokine production are suppressed by $LPA_{1/3}$ receptor antagonists, DGPP and VPC32183. Signal transduction through p42/44 MAPK, p38 MAPK and Rho kinase is involved in LPA-mediated cytokine secretion whereas LPA-induced cell motility requires p38 MAPK and Rho kinase but not p42/44 MAPK. Treatment of FLS with TNF-α increases LPA_3 mRNA expression, and correlates with enhanced LPA- or OMPT-induced cytokine production. LPA-mediated super-production of cytokines by TNF-α-primed FLS is abolished by $LPA_{1/3}$ receptor antagonists. We also report the presence of ATX in synovial fluid of RA patients. The synovial fluid-induced cell motility is reduced by $LPA_{1/3}$ receptor antagonists and ATX inhibitors. Together the data suggest that LPA_1 and LPA_3 may contribute to the pathogenesis of RA, through the modulation of FLS migration and cytokine production. The above results provide novel insights into the relevance of LPA receptors in FLS biology and as potential therapeutic targets for the treatment of RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the destruction of articular cartilage and adjacent bone tissues (Feldmann et al., 1996). The critical events in RA have been suggested to be largely orchestrated by a complex interplay of proinflammatory cytokines, chemokines, and matrix metalloproteinases both in the synovial tissue and the synovial fluid (reviewed in (Wong and Lord, 2004)). Besides inflammatory mediators, other invasive-promoting factors such as metastasis-associated protein (Senolt et al., 2006) or proliferation-mediated gene (Jang et al., 2006) have also shown to be activated in RA synovium. Interestingly, the mRNA of lysophospholipase D (lyso-PLD), also known as autotaxin (ATX), is expressed in FLS from patients with RA (Kehlen et al., 2001). ATX was originally identified as an autocrine tumor cell motility-stimulating factor and an enzyme that generates most of the extracellular lysophosphatidic acid (LPA) (Umezu-Goto et al., 2002).

LPA is a naturally occurring bioactive lipid belonging to the family of phospholipid growth factors, present in micromolar concentrations in serum and biological fluids and in higher concentrations at sites of inflammation and tumor growth (Ishii et al., 2004). LPA mediates many of its effects through interaction with a family of seven-transmembrane G-protein-coupled receptors that are encoded by the endothelial differentiation genes Edgs (Ishii et al., 2004). Five LPA receptors (LPA_{1-5}) have been characterized. Among them, LPA_{1-3} share sequence homology with one another, whereas LPA_{4-5} sequences are more divergent (Lee et al., 2006; Noguchi et al., 2003). By binding to its cognate receptors, LPA activates various signaling pathways. The cellular signaling events linking LPA to its pleomorphic activities are complex, as these receptors couple to different pertussis toxin (PTX)-sensitive and -insensitive G-proteins.

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Depending on the cell type, LPA receptors are coupled via $G_{i/o}$, Gq, and $G_{11/12}$ to multiple effector systems, such as mitogen-activated protein kinase (MAPK), adenylate cyclase, phospholipase C, and small GTPases, Rho, Rac, and Ras (reviewed in (Ishii et al., 2004)). Through interaction with LPA_1, for instance, LPA stimulates cell migration and proliferation (Yamada et al., 2004), whereas binding to LPA_2, LPA induces the synthesis of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), IL-8 and IL-6 (Palmetshofer et al., 1999). This phospholipid growth factor has been implicated in various diseases and injury states, such as angiogenesis and autoimmunity (reviewed in (Ishii et al., 2004)). Although LPA receptors are functionally expressed in a broad variety of cells, including cells found in the sub-lining of the synovial membrane (reviewed in (Takuwa et al., 2002)), little is known regarding LPA receptor biological activities and expression profile in human FLS. In RA FLS play an important role as main effector cells in joint destruction through the production of matrix metalloproteinases (MMPs), which are matrix-degrading enzymes (Firestein, 2003). FLS also migrate, invade and degrade the connective tissue of cartilage and tendon (Pap et al., 2000).

In summary, the observation that the functional responses of FLS to inflammatory stimuli resemble those induced by LPA in various cell types, that ATX mRNA is expressed by RA synoviocytes (Kehlen et al., 2001), and that RA synovial fluid contains significant amounts of the LPA precursor LPC (Fuchs et al., 2005), led us to the investigation of the expression profile and the functional responses of LPA_{1-3} receptors in FLS. We report that LPA_1, LPA_2 and LPA_3 receptor mRNA is expressed in FLS. We also provide evidence that exogenous application of LPA induces cell migration and IL-8/IL-6 secretion by FLS. Interestingly, blocking LPA receptors with $LPA_{1/3}$ receptor antagonists inhibits both LPA-induced cell motility and IL-8/IL-6

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production. Moreover, the LPA-stimulated cytokine secretion is regulated by p42/44 MAPK, p38 MAPK and Rho kinase whereas LPA-induced cell motility requires p38 MAPK and Rho kinase but not p42/44 MAPK. Additionally, we show that under an inflammatory microenvironment created by TNF-α, both the expression of LPA_3 receptor mRNA and the LPA- or OMPT-dependent secretion of IL-8/IL-6 is significantly increased. Finally we demonstrate the presence of ATX in synovial fluid of RA patients as well as the reduction of synovial fluid-induced cell motility by $LPA_{1/3}$ receptor antagonists and ATX inhibitors, which is suggestive of LPA production and LPA subsequent biological effects in human RA synovium. We therefore conclude that LPA/LPA receptor signaling may play essential role in the pathogenesis of RA.

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Materials and Methods

Reagents. 1-Oleoyl-sn-glycero-3-phosphate (LPA) and lysophosphatidylcholine (LPC) were purchased from Sigma (St. Louis, MO, USA). $LPA_{1/3}$ specific receptor antagonists, diacylglycerol pyrophosphate (DGPP) and (S)-phosphoric acid mono- (2-octadec-9-enoylamino-3- [4-(pyridine-2-ylmethoxy)-phenyl]-propyl) ester (VPC32183) were obtained from Avanti Polar Lipid Inc (Alabaster, AL, USA). ATX inhibitors 18:1 carbacyclic phosphatidic acid (18:1 ccPA, $C_{22}H_{41}O_5P$), XY-44 ($C_{22}H_{40}O_4PSNa$) and JGW-8 ($C_{20}H_{39}NaBrO_6P$) were synthesized at The University of Utah by J. Gajewiak, Y. Xu, and G. Jiang. All the compounds above were dissolved in phosphate-buffered saline (PBS) containing 0.1% fatty acid-free bovine serum albumin (BSA) from Sigma (St. Louis, MO, USA), and the aliquots were stored at –20°C. The specific LPA_2 agonist, dodecylphosphate, and LPA_3 agonist, L-sn-1-O-oleoyl-2-methyl-glyceryl-3-phosphothionate (2S-OMPT), were obtained from Biomol (Plymouth Meeting, PA, USA) and Echelon Biosciences Inc (Salt Lake City, UT, USA), respectively. Tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and tumor growth factor-β (TGF-β) were from PeproTech Inc (Rocky Hill, NJ, USA). Human IL-8 and IL-6 ELISA (Enzyme-Linked ImmunoSorbent Assay) kit were purchased from BioSource International Inc (Camarillo, CA, USA). SYBR® Green JumpStart ™ Ready Mix was obtained from Sigma (St. Louis, MO, USA). TRIzol Reagent was from Invitrogen (Burlington, ON, Canada). Inhibitors of p42/44 mitogen-activated protein kinase (MAPK) PD98059, of p38 MAPK SB203580, of Rho kinase Y27632, and of c-Jun N-terminal kinase (JNK) SP600125 were purchased from Calbiochem (San Diego, CA, USA). Antibodies to total and phosphorylated forms of p42/44 MAPK, of p38MAPK, of activating transcription factor-2 (ATF-2), and of JNK were purchased from Cell Signaling Technology (Beverly, MA,

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USA). Antibodies to LPA_1, LPA_2, and LPA_3 were obtained from MBL (Woburn, MA, USA) and Exalpha Biologicals Inc (Watertown, MA, USA). Cell culture reagents were purchased from Wisent Inc (St-Bruno, QC, Canada).

Cell culture. Human primary FLS were obtained from patients with RA, diagnosed according to the criteria developed by the American College of Rheumatology (ACR) Diagnostic Subcommittee for Arthritis, who were undergoing arthroplasty (Faour et al., 2003). Cells were maintained under standard conditions (37°C and 5% CO_2) and grown in DMEM supplemented with 10% of fetal bovine serum (FBS), penicillin (100 IU), and streptomycin (100 μM). Cells were used at passages 5 to 15.

Cell treatment. Semi-confluent cells were starved with serum-free medium for 24 h before treatment because the serum may contain up to 10 μM of LPA. At the moment of cell treatment, the culture medium was replaced with fresh serum-free medium containing various concentrations of the tested compounds as indicated in details below.

Semi-quantitative reverse transcription (RT)-PCR and real-time PCR analysis of IL-8 and LPA receptors. Cells were plated at a concentration of 5×10^4 cells/ml in 6-well plates. For analysis of IL-8 mRNA expression starved FLS were incubated with LPA (1 to 100 μM) and lysed for RNA extraction after 0.5 to 4 h. Where indicated, cells were pre-treated for 30 min with the LPA receptor antagonists DGPP (1 to 100 μM) or VPC32183 (1 to 100 μM) and incubated with LPA (50 μM) in the presence or absence of the two antagonists for 2 h prior to RNA extraction. The expression of LPA receptors was monitored in starved FLS incubated in the absence or the presence of the indicated concentrations of TNF-α (20 to 100 ng/ml) for up to 4 h prior to RNA extraction.

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Total cellular RNA was extracted using TRIzol reagent according to the instructions from the manufacturer. Total RNA (0.5-1 μg) was reverse-transcribed using random priming and Superscript II Reverse Transcriptase system (Invitrogen, Burlington, ON, Canada) following the manufacturer's guidelines. All oligonucleotides used as primers were designed to recognize sequences specific for each target cDNA. Primer sequences and PCR conditions are as follows: LPA_1 (432-bp product), sense, 5'-AAT-CGA-GAG-GCA-CAT-TAC-GG-3', antisense, 5'-TGT-GGA-CAG-CAC-ACG-TCT-AG-3'; LPA_2 (352-bp product), sense, 5'- CAT-CAT-GCT-TCC-CGA-GAA-CG-3', antisense, 5'-GGG-CTT-ACC-AAG-GAT-ACG-CAG-3'; LPA_3 (310-bp product), sense, 5'-TCG-CGG-CAG-TGA-TCA-AAA-ACA-GA-3', antisense, 5'-ATG-GCC-CAG-ACA-AGC-AAA-ATG-AGC-3'; LPA_4, (139-bp product), sense, 5'- AAA-GAT-CAT-GTA-CCC-AAT-CAC-CTT-3', antisense, 5'-CTT-AAA-CAG-GGA-CTC-CAT-TCT-GAT-3'. LPA_5, (350-bp product), sense, 5'-AGG-AAG-AGC-AAC-CAA-GCA-CAG-3, antisense, 5'-ACC-ACC-ATA-TGC-AAA-CGA-TGT-G-3'; IL-8 (562-bp product), sense, 5'-TGG-GTG-CAG-AGG-GTT-GTG-3', antisense, 5'-CAG-ACT-AGG-GTT-GCC-AGA-TTT-3'. To insure linear cDNA amplification, different amplifying cycles were tried. The experiments revealed linear amplification within 35 cycles. A total of 35 PCR cycles were run at 95°C (denaturation, 30 sec), 63°C for LPA_1, 64°C for LPA_2, 66°C for LPA_3, 60°C for LPA_4, 60°C for LPA_5, and 61°C for IL-8 (annealing, 30 sec), and 72°C (extension, 30 sec). The amount of ribosomal protein RPLP0 mRNA was used as an internal PCR control. RPLP0 (248-bp product) primer sequences are as follows: sense, 5'-GTT-GTA-GAT-GCT-GCC-ATT-G-3', antisense, 5'-CCA-TGT-GAA-GTC-ACT-GTG-C-3'. The PCR products were subjected to electrophoresis on a 0.8% agarose gel and visualized by ethidium bromide staining. Densitometry analysis was used

for band quantification using the software Alphamage 2000. The results were expressed as a ratio of the band intensity relative to the corresponding RPLP0 band obtained by amplification of the same template cDNA. Semi-quantitative real-time PCR was also conducted, using the SYBR Green PCR Master Mix kit in accordance with the manufacturer's instructions, to examine the mRNA expression of LPA_{1-3} receptors as well as to evaluate the regulation of LPA_3 mRNA expression upon TNF-α treatment. In real-time PCR experiments, we used the same primers as for RT-PCR to amplify LPA_{1-3}. The thermal cycling conditions were as follows: 95°C (initial denaturation, 3 min) followed by 40 cycles of 95°C (denaturation, 15 sec), 63°C for LPA_{1-2} and 66°C for LPA_3 (annealing, 20 sec), and 72°C (extension, 20 sec).

Wound healing assay. Cells were plated at a concentration of 5×10^4 cells/ml in 12-well plates. After routine starvation for 24 h, a plastic pipette tip was drawn across the center of the well to produce a clean wound area. Free cells were removed and the medium was replaced with serum-free medium prior to stimulation with LPA, selective LPA receptor agonists/antagonists, and synovial fluid alone or in combination with LPA receptor antagonists, ATX inhibitors, PD98059, SB203580, SP600125 or Y27632 at indicated concentrations. Immediately following scratch wounding (0 h) and after incubation for 48 h, the wound healing process was photographed with an inverted microscope (Nikon TE300). The migrated cells into the wound area were examined and monitored with the MetaMorph software.

IL-8 and IL-6 ELISA assay. Cells were plated at a concentration of 5×10^4 cells/ml in 24-well plates. After routine starvation, cells were stimulated with LPA or LPA receptor agonists (1-5 μM). Where indicated cells were pre-treated with DGPP (20 μM), VPC32183 (10 μM) or the inhibitors of p42/44 MAPK, p38 MAPK, JNK and Rho kinase for 30 min prior to

stimulation with LPA or LPA receptor agonists. To evaluate the effect of TNF-α on LPA receptor-mediated cytokine secretion, cells were either treated with TNF-α (80 ng/ml) in combination with LPA for 2 h, 8 h, and 24 h or pre-treated with TNF-α (80 ng/ml) and washed extensively prior to stimulation. Cell culture supernatants were collected and stored at –80°C until the ELISA assay was performed. IL-8 and IL-6 protein concentrations were measured, according to the manufacturer's protocol. All samples were analyzed in duplicate. Optical densities were determined using a SoftMaxPro40 plate reader at 450 nm. The results were compared with a standard curve that was generated using known concentrations (pg/ml) of IL-8 and IL-6. The results were expressed in pg/ml (5×10^4 cells).

Preparation of cell lysates and Western blotting. Cells were plated at a concentration of 5×10^5 cells/well in 6-well plates and were starved for 24 h before stimulation. To determine the activation state of p42/44 MAPK, p38 MAPK, ATF-2, and JNK, cells were exposed to LPA (5 μM) for various times (1-15 min). Where indicated, cells were pre-treated with PD98059 (25 μM), SB203580 (10 μM) and Y27632 (10 μM) for 30 min prior to stimulation. Cells were lysed in RIPA buffer [20 mM Tris/HCL (PH7.4), 1% (v/v) Triton X-100, 150 mM Nacl, 5 mM EDTA, 100 mM NaF and 1 mM Na_3VO_4]. Protein concentration was determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, U.S.A), using BSA as standard. Equal amount of proteins (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to methanol soaked immobilon PVDF membranes (Millipore corporation, Bedford, MA, USA). Primary antibody incubation was performed overnight in 5% (w/v) milk at 4 °C. The membranes were then washed three times and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature. Membranes were washed for three times and

antibody-antigen complexes were revealed using ECL® according to the manufacturer's instructions (Perkin Elmer Life Sciences, Boston, MA, USA)

Synovial fluid. This research was conducted according to the principles of the Declaration of Helsinki. Eleven synovial fluid samples were studied, originating from patients (8 women, and 3 men, with a mean age of 51.2 ± 3.4 years) with definite or classical RA according to the ACR criteria. After informed consent had been obtained, synovial fluid was collected on heparin, centrifuged to eliminate cells and debris, and frozen at –20°C. Synovial fluid (2.5 µl) was mixed with one volume of boiling Laemmli sample buffer immediately prior to electrophoresis. To examine the contribution of ATX to FLS motility, synovial fluids were dialyzed using a 100 000 Molecular Weight cut off membrane to remove free or serum albumin-bound lysophospholipids including sphingosine-1-phosphate before addition to cell culture medium (Fuchs et al., 2005; Kitano et al., 2006).

ATX DNA constructs, cell transfection and immunoblotting. The ATX cDNA was prepared and transfected into CHO2A cells as described previously (Murata et al., 1994). Cell lysates were resolved on 8% SDS-PAGE and transferred to PVDF membranes prior to Western blotting with affinity-purified anti-ATX (1/2000) antibodies (Murata et al., 1994).

Statistical analysis. Unless otherwise stated, experiments were performed in triplicates. Results presented are expressed as mean values ± SE or as representative studies. Statistical significance of the difference between treated and untreated samples was determined by analysis of variance (*t*-test). Calculations were made with the Prism software 4.0. *P* values less than 0.05 were considered statistically significant.

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Results

Messenger RNA expression of LPA_1, LPA_2, and LPA_3 in human FLS. Since the biological activity of LPA is mediated through its interaction with specific cell surface receptors, we first examined the presence of LPA receptor transcripts in primary human FLS. Using semi-quantitative RT-PCR and real-time PCR, we detected mRNA for LPA_1, LPA_2, and LPA_3 (Figure 1), but not LPA_4 or LPA_5 (data not shown), in cultured human FLS from RA patients. The most abundantly expressed receptor at the mRNA level was LPA_1, compared to LPA_2 and LPA_3.

Induction of human FLS migration by LPA via its receptors. The migration of FLS into cartilage and bone is central to RA pannus development. We therefore investigated whether LPA could directly alter the migratory behavior of these cells, using a wound healing assay. As shown in Figure 2, exogenously added LPA was able to induce migration of FLS. The migratory response mediated by LPA was strongly reduced by specific antagonists against $LPA_{1/3}$, namely DGPP (58% decrease) and VPC32183 (64% decrease). The results suggest that LPA, via LPA_1 and/or LPA_3 receptors, stimulates FLS migration.

Stimulation of IL-8 and IL-6 production by LPA in human FLS. LPA is known to induce both IL-8 and IL-6 secretion in several other cell lines (Fang et al., 2004; Saatian et al., 2006). Since infiltration of inflammatory cells into the synovium is another important characteristic of RA pathogenesis, we next investigated whether LPA could be involved indirectly in this process by regulating the production of IL-8 and IL-6 of FLS. We chose to investigate IL-8 and IL-6 since they are potent neutrophil chemoattractants involved in RA disease progression (Koch, 2005). As shown in Figure 3A, no significant IL-8 mRNA expression was detected in control starved FLS from RA patients. Upon treatment with LPA,

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however, FLS expressed IL-8 mRNA in a dose- (Figure 3A) and time- (Figure 3B) dependent manner, with a maximal induction observed at 50 μM of LPA, and at 2 h post-stimulation. The effect of LPA induction seems to be specific because IL-8 expression in FLS was not induced by a treatment with related lipids such as LPC (data not shown).

The LPA-induced IL-8 and IL-6 protein secretion was also monitored. In this series of experiments, we used lower concentrations of LPA to avoid the cytotoxic effect of LPA, observed at ≥ 10 μM on starved FLS after an incubation of 24 h. A significant release of IL-8 (Figure 3C) protein was detected with 1-5 μM LPA. LPA-stimulated IL-8 (Figure 3D) secretion continued to increase for up to 24 h, the last time point tested. LPA also stimulated IL-6 secretion in a dose- and time-dependent manner (Figure 3E and 3F). These results demonstrate that LPA is able to induce an up-regulation of IL-8 and IL-6 production in human FLS.

Since the effects of LPA are likely receptor-mediated processes, we next analyzed the impact of two specific antagonists of $LPA_{1/3}$, DGPP and VPC32183, on LPA-induced IL-8 and IL-6 production in FLS from RA patients. We observed that DGPP (Figure 4A) and VPC32183 (Figure 4B) significantly inhibit, in a concentration-dependent manner, the LPA-mediated IL-8 mRNA expression. DGPP and VPC32183 almost completely abolished IL-8 mRNA expression (up to 85%), with optimal inhibition observed at a concentration of about 20 μM for both antagonists. Application of the two antagonists also significantly blocked LPA-induced cytokine secretion, DGPP (20 μM) decreased IL-8 by 70% (Figure 4C) and IL-6 by 42% (Figure 4E), whereas VPC32183 (10 μM) inhibited IL-8 by 74% (Figure 4D) and IL-6 by 94% (Figure 4F), respectively. Taken together, these results suggest that the up-regulation of IL-8 and IL-6 production by LPA in FLS involves LPA/LPA receptor (LPA_1 and LPA_3) signaling.

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Role of LPA receptors in LPA-mediated functional responses. To more accurately distinguish the contribution of LPA_1, LPA_2, and LPA_3 receptors to LPA-mediated responses, we employed the selective LPA_2 agonist, dodecylphosphate, and LPA_3 agonist, OMPT (Figure 5) in our functional assays. The LPA_2 agonist had no effect on either cell motility (Figure 5A) or cytokine secretion (data not shown). In contrast, the LPA_3 agonist stimulated cytokine secretion (Figure 5B, C) but not cell motility (Figure 5A). Taken together, the data suggest that LPA_1 and LPA_3 may play a major role in LPA-induced FLS motility and cytokine secretion, respectively.

Involvement of the downstream signaling pathways coupled to LPA functional responses. As MAPK pathways play important role in various cellular activities, including the induction of IL-8 and IL-6 (Oz-Arslan et al., 2006), and Rho kinase regulates actin reorganization and thus the cell motility (reviewed in (Tawara and Shimokawa, 2007)), inhibitors of the p42/44 mitogen-activated protein kinase (MAPK) PD98059, of p38 MAPK SB203580, and of Rho kinase Y27632, were used to address the involvement of these signaling pathways in LPA-induced cell motility, cytokine secretion as well as their effect on the activation of p42/44 MAPK, p38 MAPK. As shown in Figure 6A, the inhibitor of p42/44 MAPK PD98059 had no significant effect on either the spontaneous or LPA-induced cell migration ($p=0.85$ for LPA *versus* LPA+PD98059 10 μM, $p=0.57$ for LPA *versus* LPA+PD98059 25 μM), whereas the inhibitor of p38 MAPK SB203580 at 10 μM decreased both spontaneous and LPA-mediated cell migration by 37% and 67%, respectively (Figure 6B). The most significant effect was observed with Rho kinase inhibitor Y27632. It blocked LPA-induced cell migration by 81% at 10 μM (Figure 6C). The results suggest a role for p38 MAPK and Rho kinase in LPA-modulated cell motility.

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Regarding cytokine production, all three inhibitors inhibited LPA-induced cytokine release (Figure 7). The inhibitor of P42/44 MAPK PD98059 (25μM) decreased LPA-induced IL-8 secretion by 78% (Figure 7A) and that of IL-6 by 83% (Figure 7D). The inhibitor of p38 MAPK SB203580 (10μM) reduced LPA-mediated IL-8 and IL-6 secretion by 66% (Figure 7B) and 67% (Figure 7E), respectively. The inhibitor of Rho kinase inhibitor Y27632 almost totally blocked LPA-stimulated IL-8 (Figure 7C) and IL-6 (Figure 7F) secretion at 10 μM, the highest concentration tested. These data suggest a role for p42/44 MAPK, p38 MAPK, and Rho in the modulation of IL-8 and IL-6 secretion by LPA.

The specificity of the signaling inhibitors was determined by assessing the activation state of p42/44 MAPK, p38 MAPK and ATF-2, a downstream target of p38. LPA enhanced the phosphorylation of p42/44 MAPK and p38 MAPK. Phosphorylation peaked at 5 min for p42/44 MAPK and 15 min for p38 MAPK (data not shown). As expected, PD98059 attenuated basal and LPA-induced phosphorylation of p42/44 MAPK (Figure 8A) but had no significant effect on LPA-induced phosphorylation of p38 MAPK and ATF-2 (Figure 8B). LPA-induced phosphorylation of p38 MAPK and ATF-2 (Figure 8B) but not that of p42/44 MAPK (Figure 8A) were blocked by the inhibitor of p38 MAPK SB203580. In this regard, the inhibition by SB203580 of LPA-induced p38 MAPK phosphorylation suggests that activation of this signaling pathway in FLS involves p38 MAPK autophosphorylation. Interestingly, the Rho kinase inhibitor Y27632 had no effect on LPA-induced activation of p42/44 MAPK but significantly diminished that of p38 MAPK (Figure 8C). These results indicate that LPA is a potent activator of p42/44 MAPK and p38 MAPK in FLS. The data also suggest that p38 MAPK may act, at least

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in part, downstream of Rho/Rho kinase to promote LPA-induced FLS motility and cytokine secretion.

We also explored the involvement of the JNK signaling pathway in LPA-induced FLS functional responses. Phosphorylated JNK was not detected after stimulation with LPA (data not shown). Furthermore, LPA-mediated IL-8 secretion and cell motility were not inhibited by the JNK inhibitor SP600125 (data not shown). The results suggest that JNK is not activated by LPA in human FLS.

Regulation of LPA_1, LPA_2, and LPA_3 receptor mRNA expression by pro-inflammatory stimuli in human FLS. Since TNF-α is a key inflammatory molecule in the RA cytokine network (Taberner et al., 2005), we also analyzed the mRNA expression profile of LPA receptors in response to TNF-α. We found that treatment with TNF-α, at concentrations of 20-80 ng/ml, up-regulated the mRNA expression of LPA_3 in a dose-dependent manner (Figure 9A). The maximal effect observed was a 3.5 ± 0.3 fold increase in LPA_3 mRNA expression, induced by 80 ng/ml of TNF-α. To further investigate the kinetics of TNF-α-induced LPA_3 expression, FLS were exposed to 80 ng/ml of TNF-α for 0.5-4 h. Semi-quantitative RT-PCR analysis showed that LPA_3 mRNA, normalized to that of RPLP0, peaked after 2 h stimulation with TNF-α and decreased thereafter (Figure 9B). On the other hand, under the same conditions, LPA_1 and LPA_2 expression was not altered by TNF-α (data not shown). It is noteworthy that no up-regulation of LPA_3 mRNA was observed in FLS treated with other cytokines, such as IL-1β and TGF-1β (data not shown). Up-regulation of LPA_3 expression by TNF-α (Figure 9A, B) was further confirmed by quantitative real-time PCR (Figure 9C). The results indicate that the expression of LPA receptors, at least that of LPA_3, can be up-regulated by TNF-α. Receptor

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expression in human FLS was below the threshold for detection by LPA_1, LPA_2 and LPA_3 antibodies (data not shown).

Effect of TNF-α on LPA-induced functional responses in human FLS. The next series of experiments was undertaken to examine the effect of a proinflammatory environment, established by a pre-treatment of FLS with TNF-α, on both of the functional experiments we performed previously, i.e., LPA- or OMPT-mediated cell migration and cytokine secretion. To monitor the effect of TNF-α, starved FLS were pre-treated with a range of TNF-α concentrations (1 to 80 ng/ml) and, after washing with serum-free medium, LPA- or OMPT-induced cell motility and cytokine secretion were determined. TNF-α alone did not show a significant effect either on spontaneous FLS migration or LPA-induced migration using our wound healing assay (data not shown). As shown previously in Figures 3 & 5, LPA or OMPT alone weakly stimulated cytokine production compared to TNF-α-primed FLS (Figure 10). However, after priming with TNF-α for 2 h, 8 h and 24 h, LPA-induced IL-8 secretion was strongly enhanced. TNF-α pre-treatment (80 ng/ml, 24 h) increased up to 38 times LPA-induced IL-8 release (Figure 10A). Moreover, LPA-induced IL-8 production was strongly enhanced after priming with a concentration of TNF-α (80 ng/ml) previously shown to up-regulate LPA_3 mRNA expression by FLS (Figures 10B and 5). The production of IL-8 (Figure 10C) and of IL-6 (Figure 10D) induced by the selective LPA_3 agonist OMPT was also super-stimulated after a pre-treatment of FLS with TNF-α. The results emphasize the potential contribution of LPA and signaling through LPA receptors in the inflamed synovium. To determine the relevance of the LPA receptor(s) to LPA-mediated enhanced cytokine production after priming with TNF-α, FLS were treated with the LPA receptor antagonists, DGPP and VPC32183. DGPP and VPC32183

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had no effect on TNF-α-mediated IL-8 or IL-6 secretion, but almost completely inhibited the enhanced secretion of cytokine induced by LPA in TNF-α-primed cells (Figure 11). The results indicate that TNF-α modulates LPA (LPA_3) receptor functional expression and responses in human FLS.

Contribution of ATX to synovial fluid-mediated FLS motility. Autotaxin **(**ATX) was originally identified as an autocrine tumor cell motility-stimulating factor and shown to be a lyso-phospholipase D (lyso-PLD), an enzyme that generates LPA from lysophospholipids such as LPC (Umezu-Goto et al., 2002). Since synovial fluid contains significant amounts of the LPA precursor LPC (Fuchs et al., 2005), we monitored the presence of ATX in synovial fluid from patients with RA. As shown in Figure 12A, using the affinity purified anti-ATX antibody, we detected the presence of the full length ATX protein in synovial fluid from patients with RA. The addition of dialyzed synovial fluid to cell culture medium (2.5%, final) strongly stimulated the motility of FLS (Figure 12B, C). To link the role of ATX to LPA production and synovial fluid-mediated FLS motility we examined with wound healing assay the effect of ccPA 18:1, a pure inhibitor of ATX with no significant agonist or antagonist activity at LPA receptors (Baker et al., 2006; Jiang et al., 2007; Xu et al., 2006), and of JGW-8 (Baker et al., 2006; Jiang et al., 2007) and XY-44 (Xu et al., 2006), two compounds that exhibit both submicromolar inhibition of ATX and submicromolar antagonist activity for four LPA receptors. As shown in Figure 12B, treatment of cells with 5 μM ccPA 18:1, JGW-8 or XY-44 diminished synovial fluid-induced FLS motility by 58%, 54% and 48%, respectively. Synovial fluid-mediated FLS motility was also inhibited by $LPA_{1/3}$ antagonists (Figure 12C). DGPP (10 μM) and VPC32183 (10 μM) reduced synovial fluid-induced cell motility was 64% and 84%, respectively. Collectively, these

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results provide strong support to the hypothesis that ATX in synovial fluid produces LPA and stimulates human FLS through activation of $LPA_{1/3}$.

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Discussion

In the present study we report several novel findings regarding LPA receptor expression, regulation, and function in FLS from RA patients. We provide direct evidence for the mRNA expression of LPA_{1-3} receptors, LPA-induced cell migration and secretion of IL-8 and IL-6 by FLS. The LPA-induced effects were shown to be driven by signaling through the $LPA_{1/3}$ receptors and regulated by p42/44 MAPK, p38 MAPK, and Rho kinase. Moreover, both LPA_3 receptor expression and LPA-induced cytokine secretion by FLS are modulated by the inflammatory cytokine TNF-α. We also demonstrate that ATX is present in synovial fluid and that ATX inhibitors or $LPA_{1/3}$ receptor antagonists can reduce the synovial fluid-induced FLS motility. To the best of our knowledge, this is the first report of the functional expression and regulation of LPA receptors in human FLS.

The main histological characteristic of RA is the hyperplasia of the synovial intimal lining cells. As a constituent of synovial pannus in RA, FLS have long been considered as key players in the aggressive invasion of cartilage and bone (Shiozawa et al., 1983). FLS are thought to migrate over the cartilage and erode into the subchondral bone, eventually resulting in the formation of erosions. However, the potential factors that direct FLS migration to form the pannus are not well known. Gilat *et al.* have reported that the expression of adhesion molecules can chemotactically guide cells with the appropriate receptors (Gilat et al., 1996). Our data show that LPA induces a strong migration of FLS, suggestive that activated LPA receptors on FLS may act directly as a driving force in the pannus invasion of cartilage in RA.

Among others, IL-8 and IL-6 have been demonstrated to affect the regulation of the signaling steps leading to neutrophil recruitment and activation (Lin et al., 2004). Previous

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studies have shown that LPA could stimulate the production of IL-8 and IL-6 by ovarian cancer, breast cancer and bronchial epithelial cells (Fang et al., 2004; So et al., 2004). In this regard, we show in the present study that serum-starved FLS do not express detectable IL-8 mRNA or secrete IL-8 in the resting state. In contrast, exogenously applied LPA strongly promotes IL-8 mRNA expression and IL-8/IL-6 secretion by FLS. Thus, LPA may contribute to the regulation and the maintenance of the inflammatory response in RA, in part through stimulation of IL-8 and IL-6 secretion by FLS. The two cytokines may subsequently increase the recruitment of neutrophils and thus promote inflammation and neovascularization on the synovium (Middleton et al., 2004).

The biological effects of LPA are mediated by one or more LPA receptors, depending on the cell type studied. Forced expression of LPA_{1-3} has reported to increase IL-8/IL-6 production in ovarian cancer cells, with LPA_2 being more efficient in stimulating IL-8 secretion (Fang et al., 2000). In contrast, in bronchial epithelial cells LPA_1 and LPA_3 were shown to be the major receptors regulating IL-8/IL-6 production (Saatian et al., 2006). In the present study we employed a pharmacological approach to identify the specific involvement of LPA receptors in the FLS responses. The selective antagonists against $LPA_{1/3}$ DGPP and VPC32183 strongly abrogated the LPA-driven cell motility, whereas the specific LPA_2 agonist, dodecylphosphate, and LPA_3 agonist, OMPT, had no effect on this function. These findings indicate that LPA_1 receptors are more efficient in inducing the LPA-driven motility of FLS. Regarding cytokine production, the selective $LPA_{1/3}$ antagonists severely diminished LPA-dependent cytokine production. In addition, the LPA_3 but not the LPA_2 specific agonist robustly stimulated cytokine secretion. Though a role for LPA_1 in LPA-induced cytokine production cannot be excluded, our

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pharmacological approach suggests that LPA_3 drives IL-8 and IL-6 secretion in FLS.

Previous studies have implicated the p38 MAPK pathway in LPA_1 receptor-mediated migration of glioma cells (Malchinkhuu et al., 2005) and Rho kinase in LPA-induced migration of airway smooth muscle cells (Hirakawa et al., 2007). Other studies also illustrated that LPA-induced IL-8 and IL-6 secretion is regulated by p38 MAPK (Saatian et al., 2006), p42/44 MAPK, and Rho kinase (Oz-Arslan et al., 2006; Saatian et al., 2006). Here we show that LPA-induced FLS motility depends on the activation of p38 MAPK and Rho but not p42/44 MAPK or JNK. We also demonstrated that signal transduction through p42/44 MAPK, p38 MAPK, and Rho kinase but not JNK is involved in LPA-dependent cytokine secretion. Our results suggest that coupling of LPA receptors to various heterotrimeric G-proteins, and thereof distinct downstream signaling pathways contribute to LPA-mediated cytokine synthesis and motility of human FLS.

In an attempt to fully understand the effects of LPA on RA synovium in vivo, we have analyzed the expression pattern of LPA receptors as well as the LPA-mediated biological responses in FLS pre-treated with TNF-α to more closely parallel the critical events that take place in the RA synovium. Although a large number of genes regulated by TNF-α and IL-1β in FLS have been identified in previous gene expression profiling studies (Taberner et al., 2005), regulation of LPA receptors by TNF-α has not been reported. In the current study we observed the selective up-regulation of LPA_3 mRNA by TNF-α, suggestive that LPA receptor(s) expression is regulated in a proinflammatory environment, such as in the inflamed synovium. TNF-α did not impact on FLS migration, either by itself or that induced by LPA (data not shown), suggesting that LPA itself is sufficient to induce a full migratory response in these cells.

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It must be pointed out that in comparison to LPA, TNF-α is a stronger stimulator of cytokine secretion. It is noteworthy that a pre-incubation of FLS with TNF-α, at a concentration found to up-regulate LPA_3 expression (80 ng/ml), for several hours prior to stimulation with LPA markedly enhanced LPA-induced IL-8 secretion (up to 38 times). Whereas TNF-α-induced cytokine secretion was not inhibited by the $LPA_{1/3}$ receptor antagonists, we observed that the enhanced LPA-induced IL-8 and IL-6 secretion after cell priming with TNF-α was totally inhibited by the $LPA_{1/3}$ receptor antagonists, DGPP and VPC38128. This raises the interesting possibility of a causal relationship between the enhanced expression of LPA_3 receptor following a treatment with TNF-α and the resultant increase in cytokine secretion. Nonetheless, the synergy of LPA and TNF-α on IL-8 and IL-6 production may be intimately associated with the inflammation of the synovium in RA. The hypothesis that LPA could be a critical mediator of cytokine secretion in RA inflammatory synovium is currently under investigation in our laboratory.

Several pathways can contribute to the production of LPA (Aoki et al., 2002). Recent studies suggest a major contribution of ATX in the production of extracellular LPA (Hama et al., 2004; Umezu-Goto et al., 2002). Moreover, LPA content is increased in the extracellular fluid of inflamed tissues (Croset et al., 2000) and upon challenge with inflammatory stimuli (Sasagawa et al., 1998). Another finding of our study is the presence of ATX protein in synovial fluid from patients with RA, of which we believe is the first report for this issue. Although the extracellular concentration of LPA in RA synovial fluid is not known, synovial fluid from RA patients contain significant amounts of LPC, which is a substrate from which ATX produces LPA (Fuchs et al., 2005). It is also interesting to point out that the levels of sphingosine-1-phosphate (S1P), another

metabolic product of ATX (Clair et al., 2003), in synovial fluid from RA patients are much higher than those found in serum and plasma (Kitano et al., 2006). Therefore it is tempting to speculate that elevated production of LPA by ATX in the joint microenvironment may contribute to the inflammation of the synovium. Our results show that RA synovial fluid strongly stimulated FLS migratory activity and this effect was inhibited by ATX inhibitors and $LPA_{1/3}$ antagonists. We thus suggest that LPA may be constantly generated from LPC by ATX in synovial fluid, building-up the concentration of LPA that lead to the subsequent responses of FLS to LPA through activation of $LPA_{1/3}$.

In summary, our data demonstrate the functional expression of LPA receptors in RA FLS, implicating this lysophospholipid in synovial cell motility and chemokine secretion such as IL-8 and IL-6. Furthermore, our data suggest that up-regulation of LPA_3 receptor expression and enhanced LPA-induced cytokine secretion by TNF-α-primed FLS would strengthen the inflammatory responses. Additionally, we report the presence of ATX/lyso-PLD in synovial fluid from patients with RA. In this context, it can be suggested that in patients with RA, enhanced production of LPA by ATX and activation of LPA receptors promotes both the migration of FLS into connective tissues and the production of cytokines. These cytokines may subsequently cause the infiltration of leukocytes and exacerbate the inflammatory response in RA synovium. Targeting LPA receptors or the production of bioactive lysophospholipids by ATX may represent innovative goals for the treatment of RA.

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Footnotes

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Legends for Figures

Fig. 1. **Expression of LPA_1, LPA_2, and LPA_3 mRNA in human FLS.** *(A) Agarose gel electrophoresis of semi-quantitative RT-PCR analysis of LPA_1, LPA_2, and LPA_3.* As a negative control, RT-PCR was performed without oligo primers. RPLP0 was used as an internal control. Experiments were repeated three times with identical results. *(B) Level of LPA receptor mRNA in human FLS relative to that of RPLP0 mRNA.* Data shown are means ± SE of three independent experiments. *(C, D) LPA_1, LPA_2, and LPA_3 mRNA expression in FLS measured using real-time PCR.* Data shown are representative of three separate experiments.

Fig. 2. **Stimulation of human FLS motility by LPA.** A clean wound area was made on a monolayer of FLS. After removing free cells, the wound was allowed to heal for 48 h in serum-free medium containing LPA (5 μM), with or without DGPP (10 μM) or VPC32183 (10 μM). The wound healing process was photographed at 0 h and at 48 h (upper panels). The data shown is representative of four separate experiments. Migrated cell numbers were expressed as percentage of non-treated cells (lower panel). Statistical comparative analyses were done between cells treated with LPA and cells treated with LPA+DGPP or LPA+VPC32183. Data shown are means ± SE of four independent experiments. (*$p < 0.05$)

Fig. 3. **LPA-induced IL-8 and IL-6 production in human FLS.** *(A) Concentration-dependent response to LPA on IL-8 mRNA expression.* Primary FLS were incubated with LPA for 2 h at various doses as indicated and lysed for RNA extraction and RT-PCR. *(B) Kinetic of LPA-induced IL-8 mRNA expression.* Cells were incubated with LPA (2.5 μM) for various time points

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as indicated before lysing for RNA extraction and RT-PCR. *(C, E) Concentration-dependent response to LPA on IL-8 (C) and IL-6 (E) secretion.* Cells were treated with LPA for 24 h at various concentrations as indicated before collecting supernatants for protein quantification. *(D, F) Kinetic of LPA-induced IL-8 (D) and IL-6 (F) secretion.* Cells were treated with LPA of indicated concentrations. Cell culture supernatants were collected at indicated time points, and cytokines were quantified by ELISA. Experiments were repeated three times and the results are displayed as representative gels (A, B) or as mean value ± SE (C-F). Statistical comparative analyses were done between samples incubated with diluents and cells treated with the indicated concentrations of LPA (C, E), and for indicated times(D, F). (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$)

Fig. 4. **Inhibition of LPA-induced IL-8 and IL-6 production by $LPA_{1/3}$ specific antagonists in human FLS.** *(A, B) Effect of DGPP and VPC32183 on LPA-induced IL-8 mRNA expression.* Human FLS were pre-treated for 30 min with DGPP (A) and VPC32183 (B) at various concentrations, as indicated, before adding LPA (50 μM) for another 2 h. Total RNA was extracted for RT-PCR. *(C, D) Effect of DGPP and VPC32183 on LPA-induced IL-8 secretion.* Cells were incubated with or without 20 μM of DGPP (C) or 10 μM of VPC32183 (D) for 30 min prior to stimulation with LPA (2.5 μM) for 24 h. *(E, F) Effect of DGPP and VPC32183 on LPA-induced IL-6 secretion.* Cells were incubated with or without 20 μM of DGPP (E) or 10 μM of VPC32183 (F) for 30 min prior to stimulation with LPA (2.5 μM) for 24 h. Results are presented as a representative electrophoresis agarose gel (upper panels A and B), or as ratios (means ± SE) of IL-8 and RPLP0 (lower panels A and B) from three independent experiments.

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For ELISA assay, experiments were repeated three times and the results are displayed as mean value ± SE (C-F). Statistical comparative analyses were done between cells treated with LPA and cells treated with LPA+DGPP (C, E) or LPA+VPC32183 (D, F). (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$)

Fig. 5. **Effect of selective LPA receptor agonists on cell motility and cytokine secretion in human FLS.** (*A) Effect of the LPA_2 agonist, dodecylphosphate, and LPA_3 agonist, OMPT, on cell motility*. After removing free cells, the wound was allowed to heal for 48 h in serum-free medium containing dodecylphosphate (5 μM) or OMPT (5 μM). LPA (5 μM) was used as a positive control. Migrated cell numbers were expressed as percentage of non-treated cells. Data shown are means ± SE of four independent experiments. *(B, C) Secretion of IL-8 and IL-6 secretion in response to OMPT*. Cells were treated with OMPT for 24 h at indicated concentrations before collecting supernatants for IL-8 (B) and IL-6 (C) measurements. Experiments were repeated three times and the results are expressed as mean value ± SE. Statistical comparative analyses were done between non-treated and OMPT-treated cells. (*$p < 0.05$; *** $p < 0.001$)

Fig. 6**. Involvement of MAPK and Rho kinase pathway in LPA-induced FLS migration.** After removing free cells, the wound was allowed to heal for 48 h in serum-free medium containing LPA (5 μM), with or without inhibitor of p42/44 MAPK PD98059 (A), p38 MAPK SB203580 (B), and Rho kinase Y27632 (C), at the indicated concentrations. Migrated cell numbers were expressed as percentage of non-treated cells. Data shown are means ± SE of three

independent experiments. Statistical comparative analyses were done between LPA and LPA+inhibitors. (*$p < 0.05$)

Fig. 7. **Involvement of p42/44 MAPK, p38 MAPK and of Rho in LPA-induced cytokine secretion.** Cells were treated with LPA (5 μM) for 24 h, in the presence or absence of p42/44 MAPK inhibitor PD98059 (A, D), p38 MAPK inhibitor SB203580 (B, E), and Rho kinase inhibitor Y27632 (C, F), at the indicated concentrations. Secreted IL-8 (A-C) and IL-6 (D-F) in cell culture supernatants were quantified by ELISA. Data shown are means ± SE of three independent experiments. Statistical comparative analyses were done between LPA and LPA+inhibitors. (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$)

Fig. 8. **Effect of p42/44 MAPK, p38 MAPK and Rho kinase inhibitors on LPA-induced activation of p42/44 MAPK and p38 MAPK.** Cells were pretreated with 25 μM PD98059, 10 μM SB203580 (A, B) and 10 μM Y27632 (C) for 30 min prior to LPA (5 μM) challenge for 15 min. Cell lysates were subjected to SDS/PAGE and samples were probed with antibodies to p-p42/44 MAPK, p42/44 MAPK, p-p38 MAPK, p38 MAPK, p-ATF-2, and ATF-2. Data shown are representative of three separate experiments.

Fig. 9. **Regulation of LPA_3 mRNA expression by TNF-α in human FLS.** *(A) Concentration-dependent response to TNF-α on LPA_3 mRNA expression.* Cells were treated with TNF-α (2 h, at various concentrations as indicated) before lysing for RNA extraction and RT-PCR. *(B) Kinetic of LPA_3 mRNA expression in response to TNF-α.* Cells were incubated with TNF-α (80 ng/ml)

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for the indicated time lengths prior to RNA extraction and RT-PCR. Results are presented as a representative electrophoresis agarose gel (upper panels) and/or as ratios (means ± SE) of LPA_3 and RPLP0 from three separate experiments (lower panels). *(C) Real-time PCR analysis of LPA_3 mRNA expression.* Cells were incubated with TNF-α (80 ng/ml) for 2 h prior to RNA extraction and real time PCR. Statistical comparative analyses were done between non-treated and TNF-α-treated cells (lower panel of A); and also between cells transiently treated with TNF-α to various time points (lower panel of B). (*$p < 0.05$)

Fig. 10. **Priming with TNF-α on LPA- or OMPT-induced cytokine secretion.** *(A) Kinetic of TNF-α pre-treatment on LPA-induced IL-8 secretion.* Cells were pre-treated with TNF-α (80 ng/ml) for different time lengths as indicated prior to stimulation with 2.5 μM LPA for 24 h. Cell culture supernatants were harvested for IL-8 measurement. *(B) Effect of increased concentrations of TNF-α on LPA-mediated IL-8 secretion.* Cells were pre-treated with TNF-α (1, 10, and 80 ng/ml) for 8 h and subsequently washed extensively before stimulation with 2.5 μM LPA for 24 h. Cell culture supernatants were harvested for IL-8 measurements. *(C, D) Effect of TNF-α priming on OMPT-induced IL-8 and IL-6 secretion.* Cells were pre-treated with TNF-α (80 ng/ml) for 8 h before adding of OMPT at indicated concentrations. Cell culture supernatants were collected for IL-8 (C) and IL-6 (D) measurement after 24 h. The results are presented as means ± SE of three separate experiments. Statistical comparative analyses were done between LPA and LPA+TNF-α (B) and between non-treated and OMPT-stimulated cells (C, D). (*$p < 0.05$; **$p < 0.01$)

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Fig. 11. **Effect of $LPA_{1/3}$ receptor antagonists on LPA-induced super production of cytokines after priming of FLS with TNF-α.** *(A, B) Effect of TNF-α on LPA-induced IL-8 secretion.* Cells were stimulated with TNF-α (80 ng/ml) for 8 h before washing and stimulation with LPA (2.5 μM) for another 24 h in the presence/absence of 20 μM of DGPP (A) and 10 μM of VPC32183 (B). *(C, D) Effect of TNF-α priming on LPA-induced IL-6 secretion.* Cells were stimulated with TNF-α (80 ng/ml) for 8 h before washing and stimulation with LPA (2.5 μM) for another 24 h in the presence/absence of 20 μM of DGPP (C) and 10 μM of VPC32183 (D). The results are presented as means ± SE of three separate experiments. Statistical comparative analyses were done between cells treated with TNF-α+LPA and TNF-α+LPA+LPA receptor antagonists. (**$p < 0.01$; *** $p < 0.001$)

Fig. 12**. Role of ATX in synovial fluid-induced FLS motility.** *(A) Expression of ATX in synovial fluids from RA patients.* Synovial fluids (2.5 μl) from 11 patients with RA was electrophoresed on an 8% SDS-PAGE and probed with anti-ATX antibody. Positive and negative controls were CHO2A cells transfected with or without pcDNA6/V5 His-ATX construct. Results shown are representative of two experiments with similar results. *(B, C) Effect of ATX inhibitors and $LPA_{1/3}$ antagonists, DGPP and VPC32183, on synovial fluid-induced cell motility.* After removing free cells, the wound was allowed to heal for 48 h in serum-free medium containing 2.5% of synovial fluid for up to 48 h in the presence/absence of ATX inhibitors ccPA, JGW-8, and XY-44 (B) or the $LPA_{1/3}$ antagonists, DGPP and VPV32183 (C) at the indicated concentrations. LPA (5 μM) was used as a positive control. Migrated cell numbers were expressed as percentage of non-treated cells. Data shown are means ± SE of four

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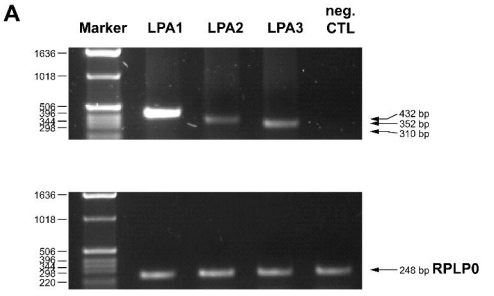
independent experiments. Statistical comparative analyses were done between cells treated with synovial fluid and synovial fluid+ATX inhibitors or synovial fluid+$LPA_{1/3}$ receptor antagonists.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Marker
LPA1
LPA2
LPA3
neg. CTL
1636
1018
506
396
344
298
432 bp
352 bp
310 bp
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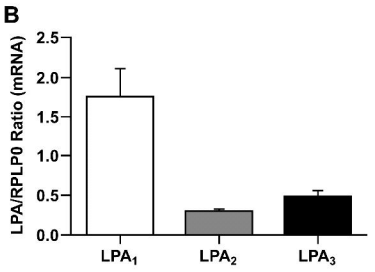
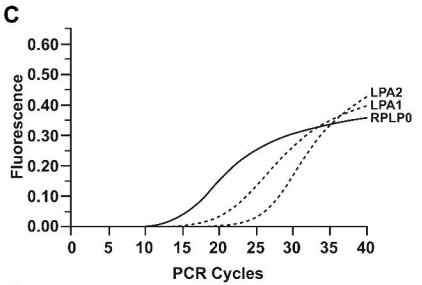
1018
506
396
344
298
220
248 bp RPLP0


B
LPA/RPLP0 Ratio (mRNA)
2.5
2.0
1.5
1.0
0.5
0.0
LPA1
LPA2
LPA3


C
Fluorescence
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
5
10
15
20
25
30
35
40
PCR Cycles
LPA2
LPA1
RPLP0

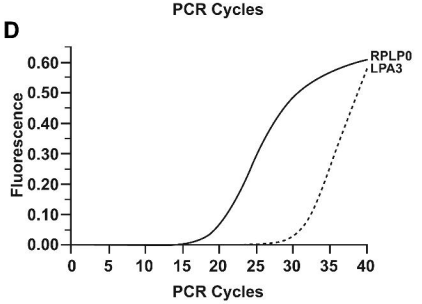
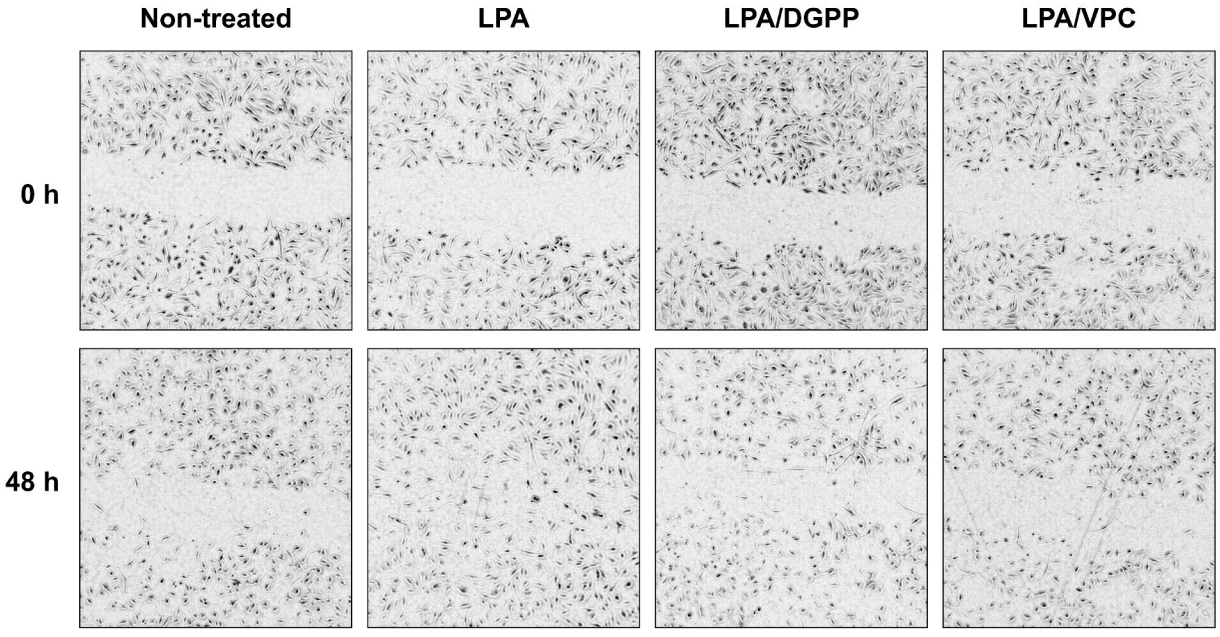

D
Fluorescence
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
5
10
15
20
25
30
35
40
PCR Cycles
RPLP0
LPA3

Figure 1


Non-treated
LPA
LPA/DGPP
LPA/VPC
0 h
48 h

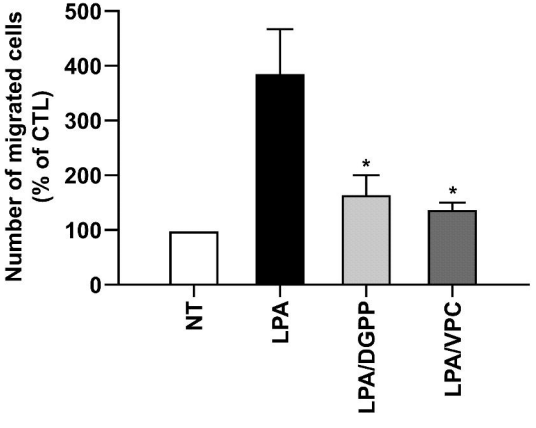

Number of migrated cells
(% of CTL)
500
400
300
200
100
0
*
*
NT
LPA
LPA/DGPP
LPA/VPC

Figure 2

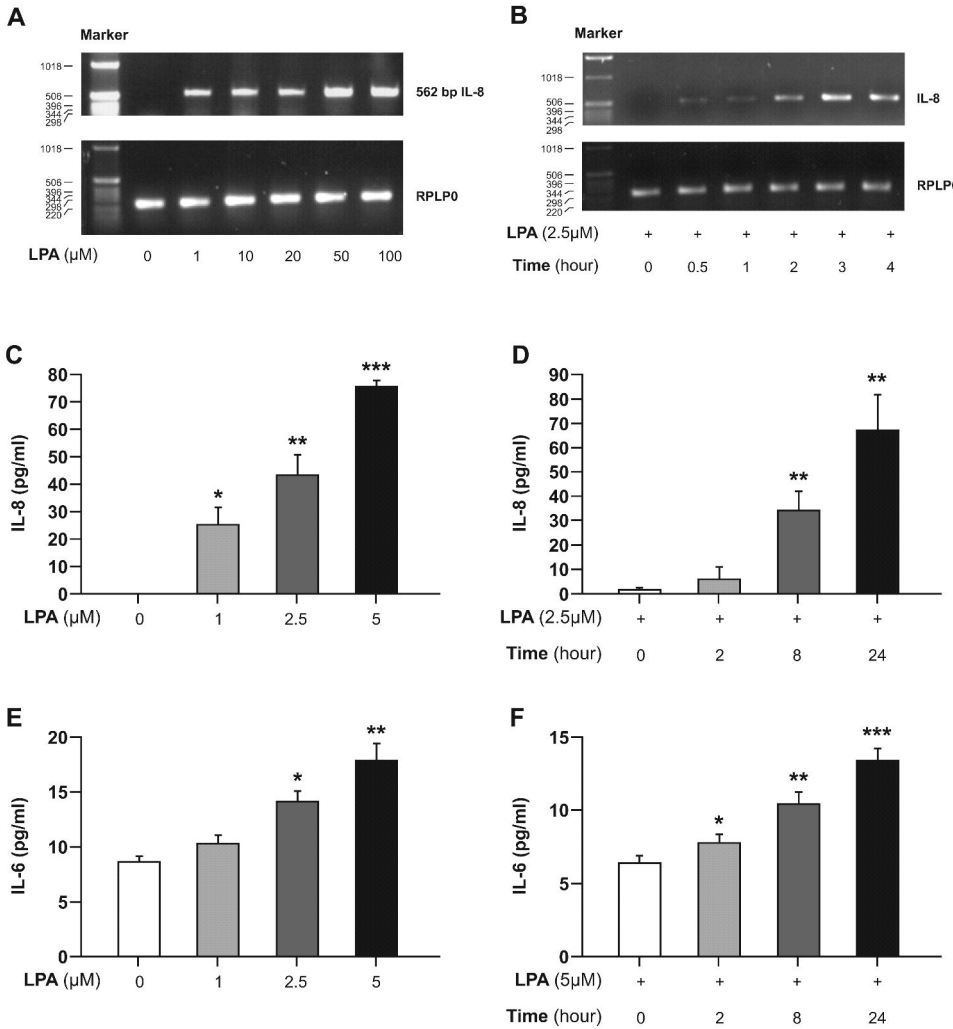

A
Marker
1018
506
396
344
298
562 bp IL-8
RPLP0
LPA (μM) 0 1 10 20 50 100
B
Marker
IL-8
RPLP0
LPA (2.5μM) + + + + + +
Time (hour) 0 0.5 1 2 3 4
C
IL-8 (pg/ml)
LPA (μM) 0 1 2.5 5
D
IL-8 (pg/ml)
LPA (2.5μM) + + + +
Time (hour) 0 2 8 24
E
IL-6 (pg/ml)
LPA (μM) 0 1 2.5 5
F
IL-6 (pg/ml)
LPA (5μM) + + + +
Time (hour) 0 2 8 24

Figure 3

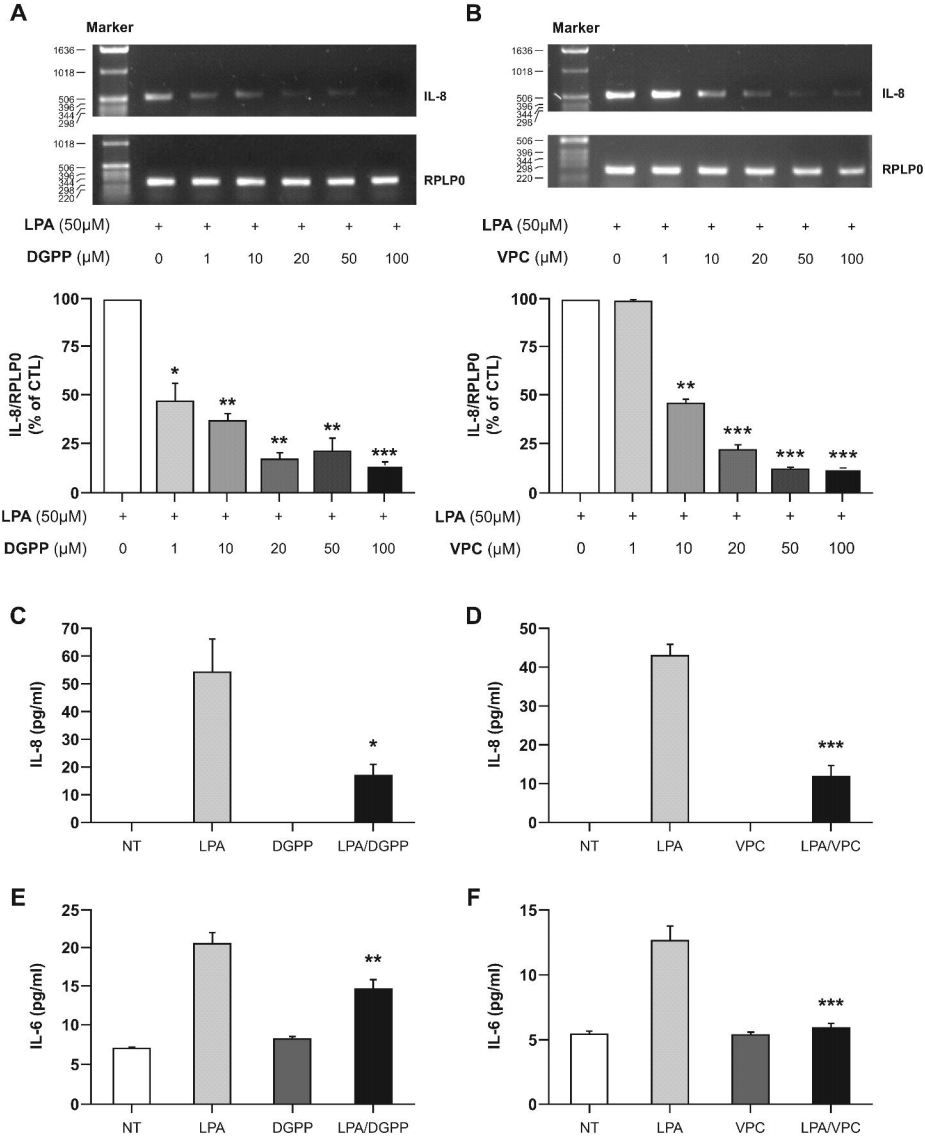

A
Marker
1636
1018
506
396
344
298
IL-8
1018
506
396
344
298
220
RPLP0
LPA (50μM) + + + + + +
DGPP (μM) 0 1 10 20 50 100
IL-8/RPLP0 (% of CTL)
100
75
50
25
0
*
**
**
**
***
LPA (50μM) + + + + + +
DGPP (μM) 0 1 10 20 50 100
B
Marker
1636
1018
506
396
344
298
IL-8
506
396
344
298
220
RPLP0
LPA (50μM) + + + + + +
VPC (μM) 0 1 10 20 50 100
IL-8/RPLP0 (% of CTL)
100
75
50
25
0
**
***
***
***
LPA (50μM) + + + + + +
VPC (μM) 0 1 10 20 50 100
C
IL-8 (pg/ml)
70
60
50
40
30
20
10
0
*
NT
LPA
DGPP
LPA/DGPP
D
IL-8 (pg/ml)
50
40
30
20
10
0
***
NT
LPA
VPC
LPA/VPC
E
IL-6 (pg/ml)
25
20
15
10
5
0
**
NT
LPA
DGPP
LPA/DGPP
F
IL-6 (pg/ml)
15
10
5
0
***
NT
LPA
VPC
LPA/VPC

Figure 4

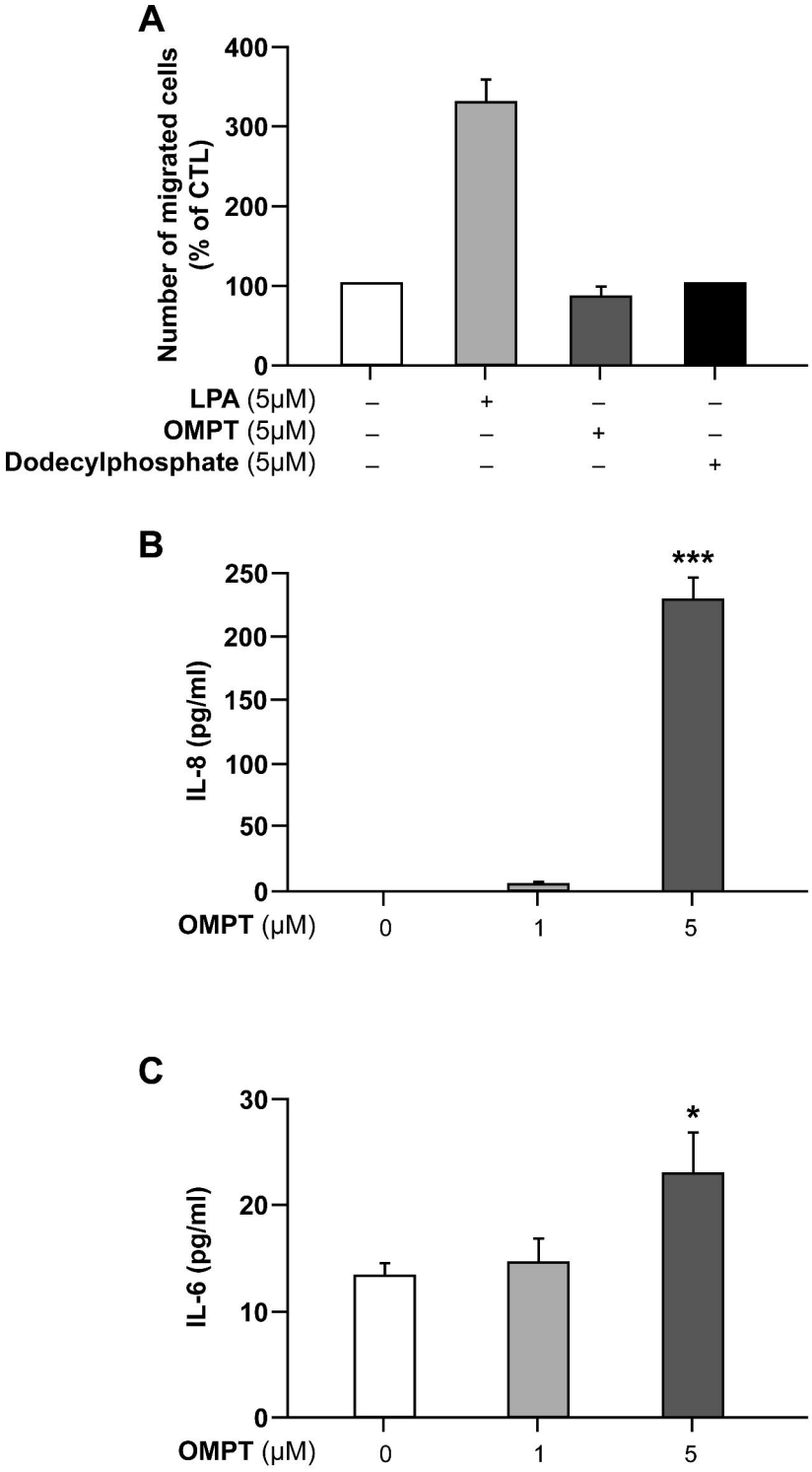
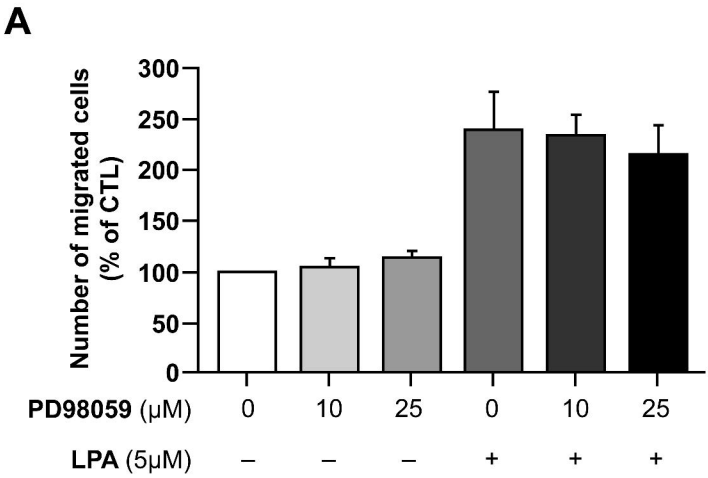
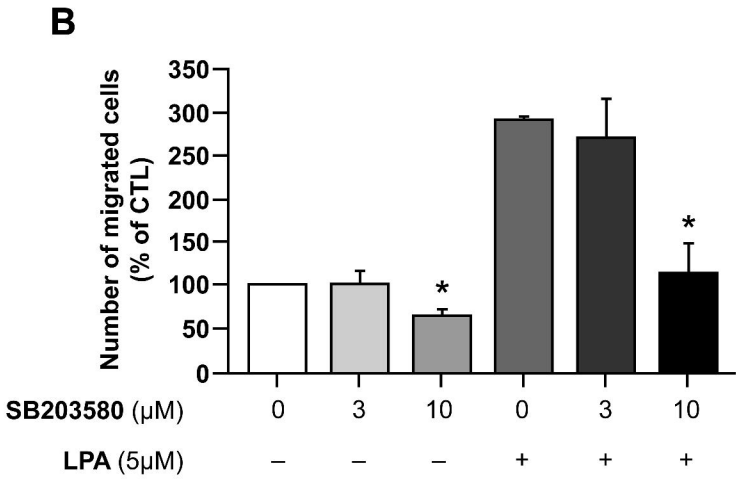

A
Number of migrated cells (% of CTL)
400
300
200
100
0
LPA (5μM) – + – –
OMPT (5μM) – – + –
Dodecylphosphate (5μM) – – – +
B
IL-8 (pg/ml)
250
200
150
100
50
0
***
OMPT (μM) 0 1 5
C
IL-6 (pg/ml)
30
20
10
0
*
OMPT (μM) 0 1 5

Figure 5


A
Number of migrated cells (% of CTL)
300
250
200
150
100
50
0
PD98059 (μM) 0 10 25 0 10 25
LPA (5μM) – – – + + +


B
Number of migrated cells (% of CTL)
350
300
250
200
150
100
50
0
*
*
SB203580 (μM) 0 3 10 0 3 10
LPA (5μM) – – – + + +

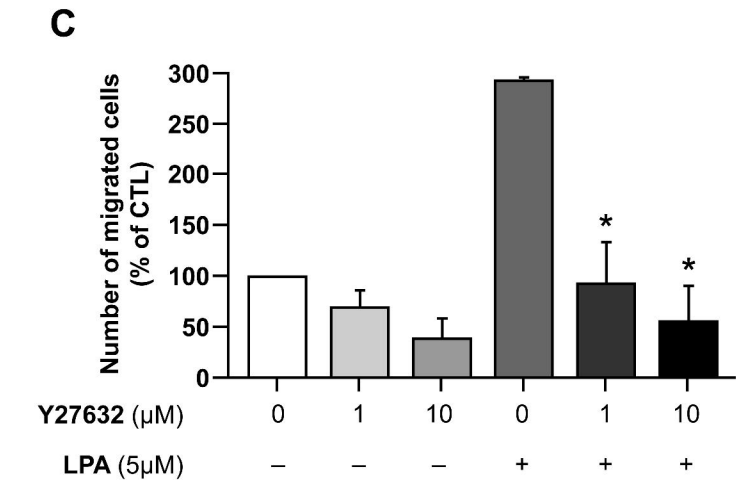

C
Number of migrated cells (% of CTL)
300
250
200
150
100
50
0
*
*
Y27632 (μM) 0 1 10 0 1 10
LPA (5μM) – – – + + +

Figure 6

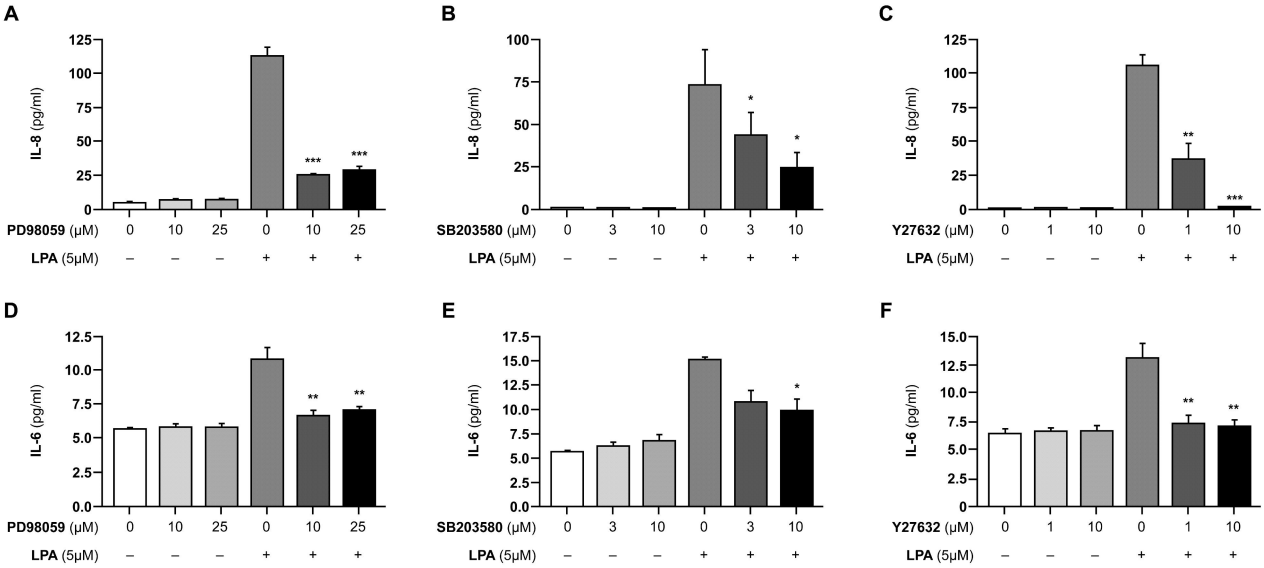

A
IL-8 (pg/ml)
125
100
75
50
25
0
***
***
PD98059 (μM) 0 10 25 0 10 25
LPA (5μM) – – – + + +
B
IL-8 (pg/ml)
100
75
50
25
0
*
*
SB203580 (μM) 0 3 10 0 3 10
LPA (5μM) – – – + + +
C
IL-8 (pg/ml)
125
100
75
50
25
0
**
***
Y27632 (μM) 0 1 10 0 1 10
LPA (5μM) – – – + + +
D
IL-6 (pg/ml)
12.5
10.0
7.5
5.0
2.5
0.0
**
**
PD98059 (μM) 0 10 25 0 10 25
LPA (5μM) – – – + + +
E
IL-6 (pg/ml)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
*
SB203580 (μM) 0 3 10 0 3 10
LPA (5μM) – – – + + +
F
IL-6 (pg/ml)
15.0
12.5
10.0
7.5
5.0
2.5
0
**
**
Y27632 (μM) 0 1 10 0 1 10
LPA (5μM) – – – + + +

Figure 7

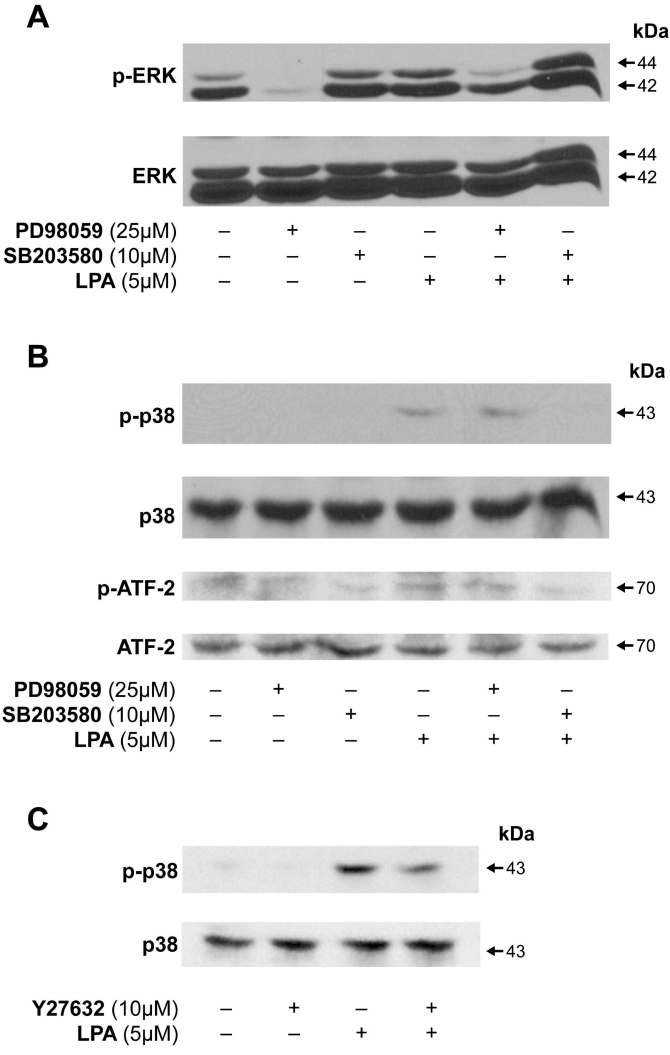

A
kDa
p-ERK
44
42
ERK
44
42
PD98059 (25μM) – + – – + –
SB203580 (10μM) – – + – – +
LPA (5μM) – – – + + +
B
kDa
p-p38
43
p38
43
p-ATF-2
70
ATF-2
70
PD98059 (25μM) – + – – + –
SB203580 (10μM) – – + – – +
LPA (5μM) – – – + + +
C
kDa
p-p38
43
p38
43
Y27632 (10μM) – + – +
LPA (5μM) – – + +

Figure 8

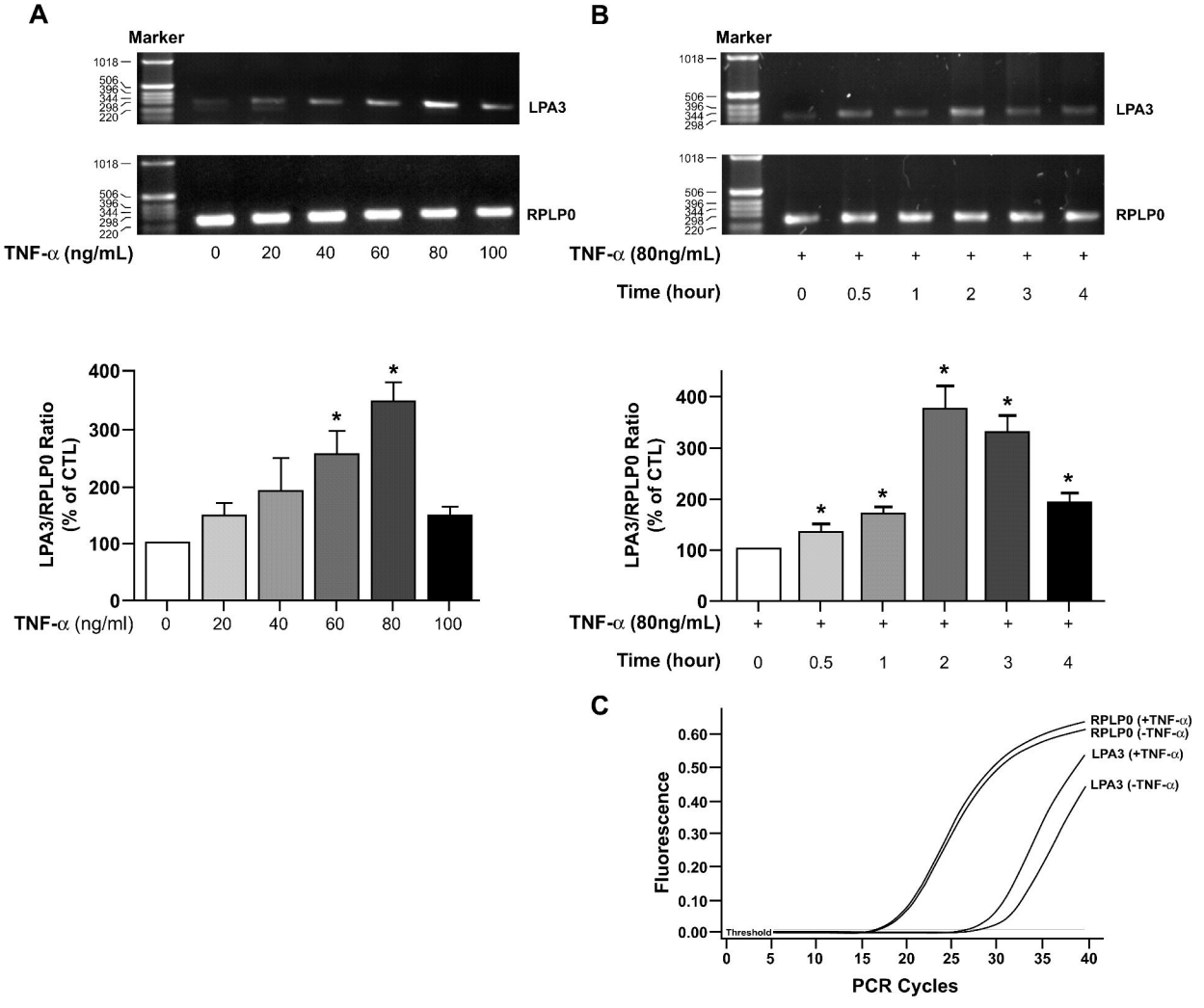

A
Marker
1018
506
396
344
298
220
LPA3
RPLP0
TNF-α (ng/mL) 0 20 40 60 80 100
LPA3/RPLP0 Ratio (% of CTL)
400
300
200
100
0
TNF-α (ng/ml) 0 20 40 60 80 100
B
Marker
TNF-α (80ng/mL) + + + + + +
Time (hour) 0 0.5 1 2 3 4
C
Fluorescence
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Threshold
0 5 10 15 20 25 30 35 40
PCR Cycles
RPLP0 (+TNF-α)
RPLP0 (-TNF-α)
LPA3 (+TNF-α)
LPA3 (-TNF-α)

Figure 9

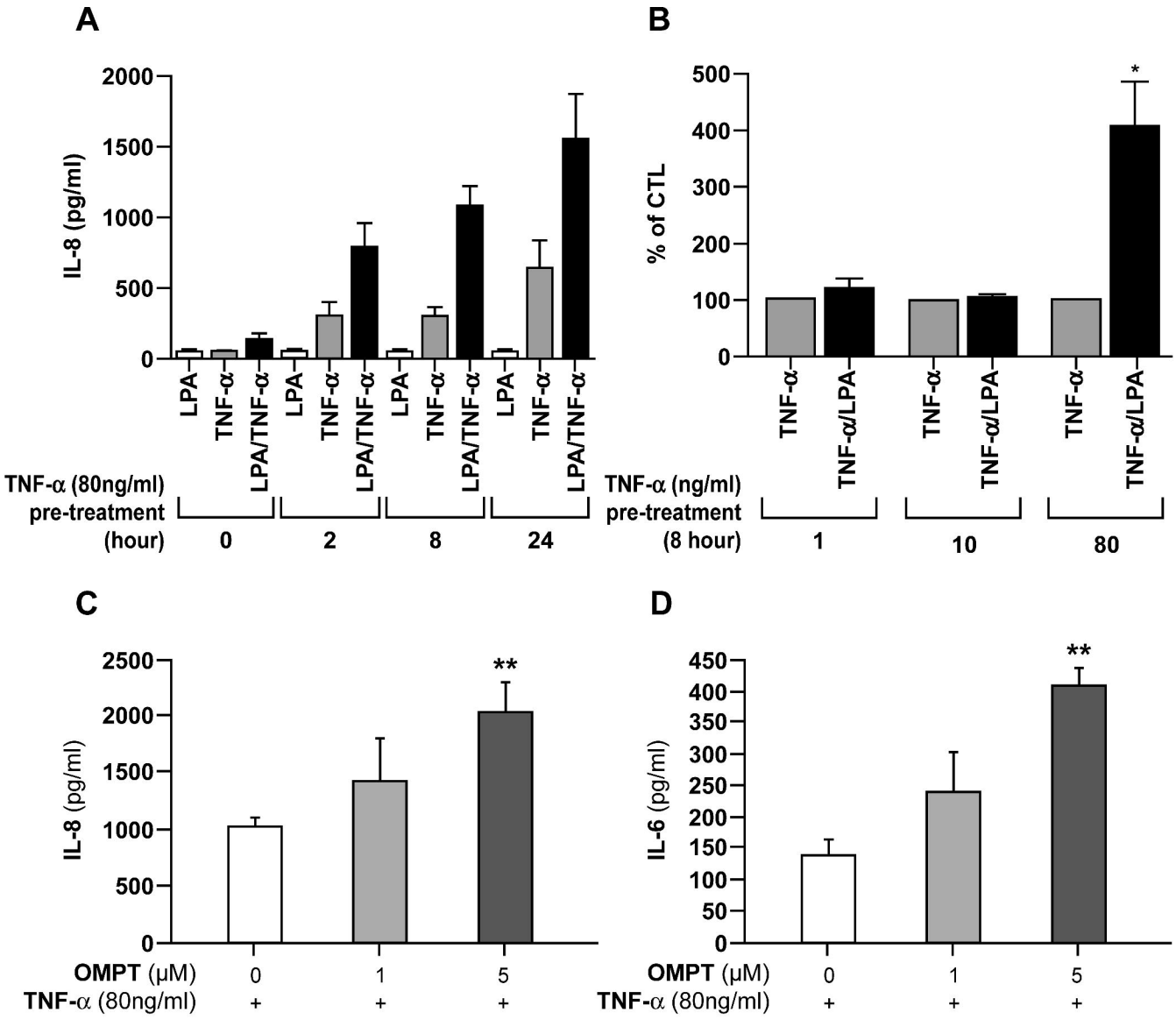

A
IL-8 (pg/ml)
2000
1500
1000
500
0
LPA
TNF-α
LPA/TNF-α
TNF-α (80ng/ml)
pre-treatment
(hour)
0
2
8
24
B
% of CTL
500
400
300
200
100
0
*
TNF-α
TNF-α/LPA
TNF-α (ng/ml)
pre-treatment
(8 hour)
1
10
80
C
IL-8 (pg/ml)
2500
2000
1500
1000
500
0
**
OMPT (μM)
TNF-α (80ng/ml)
0
1
5
+
D
IL-6 (pg/ml)
450
400
350
300
250
200
150
100
50
0
**
OMPT (μM)
TNF-α (80ng/ml)
0
1
5
+

Figure 10

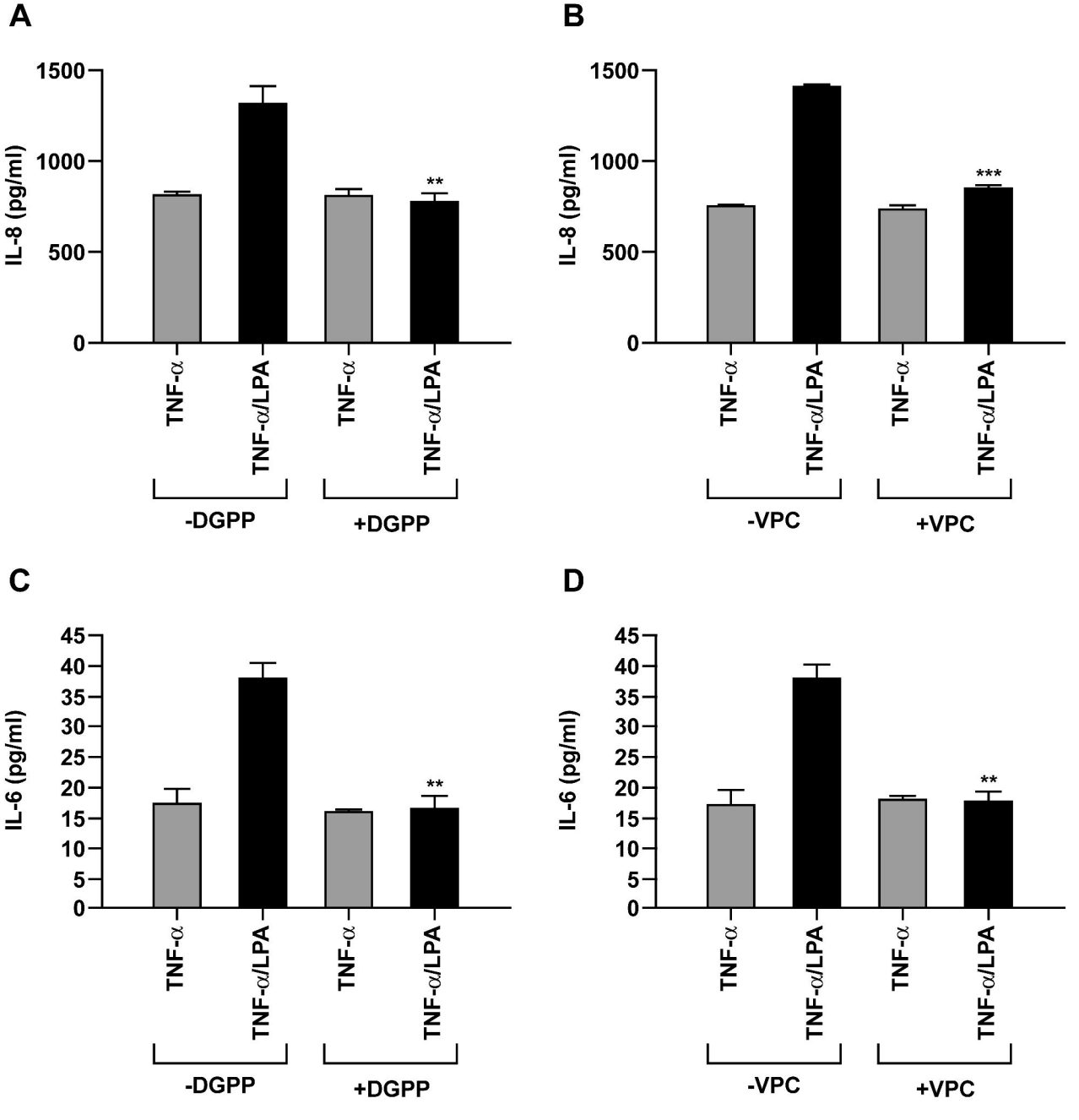
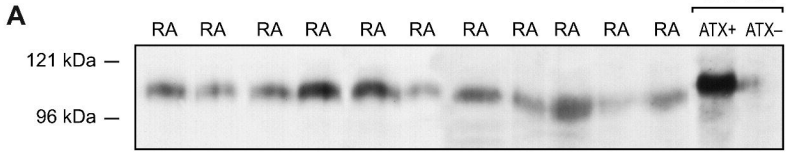
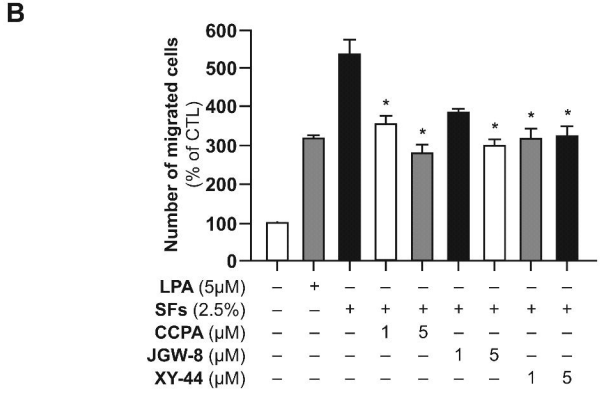

A
1500
1000
500
0
IL-8 (pg/ml)
**
TNF-α
TNF-α/LPA
TNF-α
TNF-α/LPA
-DGPP
+DGPP
B
1500
1000
500
0
IL-8 (pg/ml)
***
TNF-α
TNF-α/LPA
TNF-α
TNF-α/LPA
-VPC
+VPC
C
45
40
35
30
25
20
15
10
5
0
IL-6 (pg/ml)
**
TNF-α
TNF-α/LPA
TNF-α
TNF-α/LPA
-DGPP
+DGPP
D
45
40
35
30
25
20
15
10
5
0
IL-6 (pg/ml)
**
TNF-α
TNF-α/LPA
TNF-α
TNF-α/LPA
-VPC
+VPC

Figure 11


A
CHO 2A
RA RA RA RA RA RA RA RA RA RA RA ATX+ ATX–
121 kDa
96 kDa


B
Number of migrated cells (% of CTL)
600
500
400
300
200
100
0
LPA (5μM) – + – – – – – – –
SFs (2.5%) – – + + + + + + +
CCPA (μM) – – – 1 5 – – – –
JGW-8 (μM) – – – – – 1 5 – –
XY-44 (μM) – – – – – – – 1 5

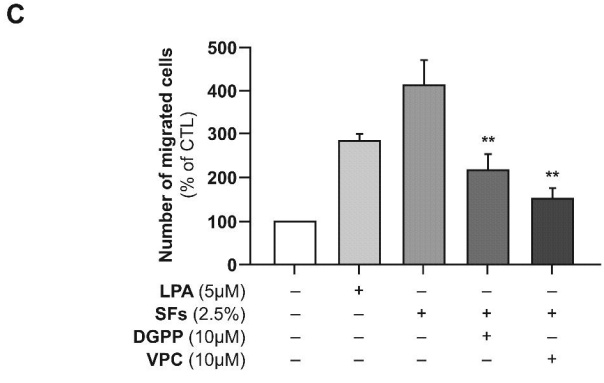

C
Number of migrated cells (% of CTL)
500
400
300
200
100
0
LPA (5μM) – + – – –
SFs (2.5%) – – + + +
DGPP (10μM) – – – + –
VPC (10μM) – – – – +

Figure 12