Advances in Rheumatology

RESEARCH

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PLCL1 regulates fibroblast-like synoviocytes inflammation via NLRP3 inflammasomes in rheumatoid arthritis

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Abstract

Background: Phospholipase C-like 1 (PLCL1), a protein that lacks catalytic activity, has similar structures to the PLC family. The aim of this research was to find the function and underlying mechanisms of PLCL1 in fibroblast-like synoviocyte (FLS) of rheumatoid arthritis (RA).

Methods: In this study, we first analyzed the expression of PLCL1 in the synovial tissue of RA patients and K/BxN mice by immunohistochemical staining. Then silencing or overexpressing PLCL1 in FLS before stimulating by TNF-α. The levels of IL-6, IL-1β and CXCL8 in FLS and supernatants were detected by Western Blot (WB), Real-Time Quantitative PCR and Enzyme Linked Immunosorbent Assay. We used INF39 to specifically inhibit the activation of NLRP3 inflammasomes, and detected the expression of NLRP3, Cleaved Caspase-1, IL-6 and IL-1β in FLS by WB.

Result: When PLCL1 was silenced, the level of IL-6, IL-1β and CXCL8 were down-regulated. When PLCL1 was overexpressed, the level of IL-6, IL-1β and CXCL8 were unregulated. The previous results demonstrated that the mechanism of PLCL1 regulating inflammation in FLS was related to NLRP3 inflammasomes. INF39 could counteract the release of inflammatory cytokines caused by overexpression of PLCL1.

Conclusion: Result showed that the function of PLCL1 in RA FLS might be related to the NLRP3 inflammasomes. We finally confirmed our hypothesis with the NLRP3 inhibitor INF39. Our results suggested that PLCL1 might promote the inflammatory response of RA FLS by regulating the NLRP3 inflammasomes.

Keywords: PLCL1, Rheumatoid arthritis, Fibroblast-like synoviocytes, Inflammatory cytokines, NLRP3 inflammasomes

Introduction

Rheumatoid arthritis (RA), a usual autoimmune disease with chronic and worsening joint destruction, is a disease that ends up being highly disabled by joint deformity and loss of function (Wang et al. [1]). The cause of RA is still undefined now. Research suggests that the pathogenesis of RA is related to genetics and other factors such as smoking, obesity, stress, neuronal suppression and female hormones. [2]. The nosogenesis of RA involves various cell types in which fibroblast-like synoviocyte (FLS) located in synovial joints serve as a vital role in pathological processes like inflammation, bone destruction and formation pannus (Mu et al. [3]). RA FLS in synovial effusion, arthritis and cartilage damage, which could also excrete kinds of inflammatory cytokines, chemokine, metalloproteinase and cathepsins, eventually causing chronic, progressive and irreversible damage of bone and

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cartilage (de Molon et al. [4]; Hu et al. [5]). Activation of RA FLS are mainly caused by inflammatory cytokines [6]. Cytokines execute lots of key physiological processes like differentiation, inflammation, cell growth, tissue repair, proliferation and the influence of the immune response (Schellekens et al. [7]). They are known to respond to FLS and various immune cells in the joint cavity of RA patients. The classical inflammatory cytokines playing pathogenic roles in RA are the IL-6, IL-1 family, TNF-α, CXCL8, IFN-γ and et al. (Mateen et al. [8]).

The animal model of arthritis we used in this experiment is the K/BxN mice. The first generation of mice born to hybrid C57Bl/6 or B10. BR mice carrying the KRN transgene heterozygously and NOD/Lt mice carrying the Ag7 allele homozygously were the K/BxN mice. K/BxN mice can spontaneously produce anti-glucose-6-phosphate isomerase autoantibodies (Anti-GPI Abs) in vivo. (Matsumoto et al. [9]). Accumulation of GPI itself in the joint articular surface, and deposition of Anti-GPI Abs amplified the complement cascade specifically in the articular tissues. The immune complexes attract and activate neutrophils and mast cells through Fc receptor binding and through activation of complement factors from the initial part of the alternative complement pathway. Complement factor C3b might bind the immune complexes and, in combination with factor B, support assembly of C3 and C5 convertases that cleave complement factor C5 to C5a and C5b. C5a, which binds to C5a receptor on neutrophils and mast cells, has potent chemotactic and degranulation activities. The activated neutrophils and mast cells release a variety of cytokines, including IL-1β and TNF-α, which play crucial roles in the development of arthritis (Ditzel [10]). About 3 weeks after birth, the first sign of arthritis develop in K/BxN mice, and pathology manifests initially in the peripheral joints with synovial hyperplasia, pannus formation and finally cartilage and bone destruction [11]. We used five-week-old K/BxN mice to experiment, because their arthritis was well developed at that moment.

In a previous new genome-wide association study with meta-analysis of rheumatoid arthritis (RA) by our team, we identified five unprecedented susceptibility loci for RA. They are the IL12RB2, PLCL1, CCR2, TCF7 and IQGAP1, respectively (Leng et al. [12]). Among others, phospholipase C-like 1 (PLCL1), a protein that lacks catalytic activity, has similar structures to the PLC family (Matsuda et al. [13]). Mutations of the amino acids within the catalytic domain of PLCL1 could change PIP2 to inositol 1, 4, 5-trisphosphate and diacylglycerol (Yoshida et al. [14]). It can inhibit significant PLC signalling downstream of Gq-protein coupled receptors and cannot weaken IP3-dependent $Ca2^{+}$ release from the endoplasmic reticulum (Harada et al. [15]; Muter et al. [3]). In view of function, it always serves as a scaffolding protein to bind phosphatases 1 and 2A as well as serine/threonine protein kinase, which transmits intrastitial signals downstream of phosphoinositide 3-kinase (Fujii et al. [16]; Sugiyama et al. [17]). PLCL1 was initially found as a brand new gene to female hip size Hip bone size (Liu et al. [18]). Recently, XIONG et al. found that PLCL1/uncoupling protein 1 (UCP1)-mediated lipid browning accelerates lipid metabolism of tumour cell and inhibits tumour progression. From the perspective of mechanism, PLCL1 regulates the protein stability of UCP1 by affecting the ubiquitination exent of UCP1 and thus regulates the lipid metabolism of tumour cells (Xiong et al. [19]). However, there are no research articles on PLCL1 in RA. The major diseased cells-FLS of RA will have tumour-like cell characteristics under inflammatory conditions of arthritis. In order to investigate the functional mechanism of PLCL1 in FLS of RA, we conducted a series of experiments built on FLS, which may help research the pathogenesis of RA and be the novel RA biomarker.

Materials and methods

Synovial tissue of human

Patients with RA (n = 6, aged 39–69) who were undergoing a joint replacement or synovectomy at the First Affiliated Hospital of Anhui Medical University have been diagnosed according to the 1987 American College of Rheumatology revised criteria. In addition, the patients with OA (n = 6, aged 39–69) were also from the Second Affiliated Hospital of Anhui Medical University. All RA cases were newly active patients. This study plan was agreed by the institutional Ethics committee of Anhui Medical University (No.20200872) and written informed consent was signed by both the patients and the control group. In this case we collected their synovial tissue according to the guidelines of the institution.

The K/BxN arthritis model

The arthritis model used in this research was the K/BxN mice. With the consent of Professors Christophe Benoist and Diane Mathis of Harvard Medical School, we introduced the KRN mice to construct the K/BxN model. Non-obese diabetic (NOD) mice were purchased from Gempharmatech Co, LTD. We mixed 1 KRN male mouse and 3 NOD female mice in each cage, and the first-generation mice of KRNxNOD were the K/BxN mice. K/BxN mice developed redness and swelling spontaneously in the joints three weeks after birth. We removed five-week-old K/BxN mice's ankle joints for experiment. The K/BxN mouse was a spontaneous arthritis model. We used five-week-old KRN mice (n = 6) as controls for K/BxN mice (n = 6). We strictly abide by the Administration of Experimental Animal regulations issued by China's State

Science and Technology Commission. In addition, Experimental projects related to animals have been agreed by the Animals Use and Care Committee (No.20200863).

Histopathology and immunohistochemical staining

Synovial tissues of human and ankle joints of mice were immersed in 10% paraformaldehyde for 1 d. In addition, we decalcified the ankle joints of mice. We soaked the ankle joints of mice with EDTA decalcification solution (Beyotime, China) and replaced it once a day for 30 days. Synovial tissue and the decalcified ankle joints were then embedded in paraffin, sliced (5 μm thick), and stained with hematoxylin and eosin (H&E). Paraffin-embedded tissue (5 μm thick) was immunohistochemically stained for PLCL1, including dehydration, dew axing, and hydration. Antigen extraction and goat serum blocking were performed. Then, tissue was incubated with anti-PLCL1 antibodies (Bioss, China) overnight and incubated with secondary antibodies. Subsequently, sections were observed under a microscope (Olympus, Japan) at × 20 magnification to assess the difference in PLCL1 expression in synovial tissues.

Cell culture and transfection

The synovial tissues were cut into pieces and placed in cell culture flasks immediately after collection to derived FLS. FLS was cultured in high-glucose DMEM medium (Hyclone, USA) containing 20% fetal bovine serum (PAN, Germany) and 1% penicillin–streptomycin-amphotericin B (Beyotime, China) in an incubator at a temperature of 37 °C with 5% CO2. FLS from the 3th to the 5th generation were used in the subsequent experiments. When the cell density reached 90%, they were digested with 0.25% trypsin (Beyotime, China) and seeded in 6-well plates for the experiment. The PLCL1-RNAi (human) sense strand is 5′-GCUCAGGAAUAUCGGU CUUTT-3′ and the antisense strand is 5′-GCUCAGGAAUAU CGGUCUUTT-3′. Transfection was performed using RNAFIT transfection reagent (Hanbio, China) according to the experimental instructions. After 6 h, the previous medium was replaced with fresh medium, and then TNF-α (10 ng/ml) (Peprotech, USA) was added for 48 h. These FLS were then subjected to a series of analysis. PLCL1-pcDNA3.1 of the human overexpression plasmid is from Hanbio (Shanghai, China). Before transfection, the FLS were cultured in six-well plates. Transfection was performed using Lipfit3.0 transfection reagent (Hanbio, China) as to the experimental instructions. After 6 h of transfection, we replaced the previous medium with fresh medium and then incubated it with TNF-α (10 ng/ml) for an additional 48 h. Afterwards, we performed a series of subsequent experiments.

Immunofluorescence (IF)

We estimated PLCL1 protein expression in FLS before and after stimulation with TNF-α (10 ng/ml). Meanwhile, we also examined the Vimentin to identify FLS. Briefly, FLS were incubated simultaneously with the mouse-derived PLCL1 antibody (Santa, USA) and rabbit-derived Vimentin (Danvers, USA), followed by corresponding secondary antibodies. Finally, we used Confocal Laser Scanning Microscope (Leica TCS SP8) to detect and analyze cells.

Western blot (WB)

We collected protein in FLS and synovial tissues using RIPA (Beyotime, China) lysis buffer with 1% protease inhibitor cocktail (Beyotime, China). Protein concentration was determined by the BCA kit (Beyotime, China). Then we took 20 ~ 30 μg protein supernatant mixed with upper sample buffer (Beyotime, China), boiled 10 min. Samples after 10% SDS–polyacrylamide gel electrophoresis transferred protein to PVDF membrane by wet transfer (200 mA, 50 ~ 60 min). PVDF membranes were immersed in a blocking solution containing 5% skim milk powder and blocked for 2 h at room temperature. After drift, they were then incubated overnight for 4 ℃ using PLCL1 antibody (1: 2 000) (Abcam, UK), IL-6 antibody (1: 2 000) (Bioworld, China), IL-1β antibody (1: 2 000) (Wanleibio, China), NLRP3 (1: 2 000) (Wanleibio, China), Caspase-1/Cleaved Caspase-1 antibody (1:1 000) (Wanleibio, China), β-actin antibody (1: 2 000) (Bioss, China), then incubated with secondary antibody (1: 2 000) (Bioss, China). Finally, the enhanced chemiluminescence kit (Thermo Scientific, USA) was used to display the protein bands. The expression of protein was quantified by ImageJ software.

Real-Time quantitative PCR (RT-QPCR)

Total RNA of FLS and synovial tissue was extracted by the TRIZOL reagent (Invitrogen, USA). RNA was reverse transcribed to cDNA by the 5 × Prime Script RT Master Mix (AccurateBiology, China). The total volume of PCR reactions of 20 μl includes a 10 μl power SYBR green PCR master mix (AccurateBiology, China), 100 ng cDNA and 0.2 mol /L of righteous and antisense primers. Cycle parameters: 95 ℃ 5 min predenaturation, 95 ℃ denaturation of 1 min, 60 ℃ multiplaturation and extension of 30 s. The last 72 ℃ was extended for 5 min. β-acting was used as a reference control, and quantification was performed using the comparative Ct method (2- ΔΔCt). Primer sequences used in this experiment are available for view (Table1). The primers are all from the Chinese Sangon Biotech Company.

Table 1 Primer sequences for human genes in this study

Gene	Forward sequence	Reverse sequence
PLCL1	CTTGAATCCACAGGACTTTTGG	TAGAATGCCCTTTGTATTTGCG
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC
IL-6	AGACAGCCACTCACCTCTTC	AGTGCCTCTTTGCTGCTTTC
IL-8	TCCTAGTTTGATACTCCCAGTC	ACAAGTTTCAACCAGCAAGA
IL-1β	GCCAGTGAAAYGATGGCTTATT	AGGAGCACTTCATCTGTTTAGG
MMP-3	ACTCGAGTCACACTCAAGGG	ACAAGGTGCAAGCTAAGCAG
MMP-9	GACAAGCTCTTCGGCTTCTG	CAAAGTTCGAGGTGGTAGCG

Cytokine assay by enzyme-linked immunosorbent assay (ELISA)

Supernatants of FLS were collected. To detect the level of IL-6, CXCL8 and IL-1β in the supernatant following the instructions of the ELISA kit (Jymbio, China).

Statistical analysis

All the data was performed using the SPSS 23.0 statistical software. Each experiment was repeated at least three times, and the results were presented as mean ± SD. Differences were analyzed by paired T-test within intra-group. ANOVA was used to compare the differences among groups. $P<0.05$ was regarded to be statistically significant.

Result

The expression of PLCL1 was up-regulated in RA FLS

First, we screened the five susceptibility loci by RT-QPCR and found that PLCL1 was the most significant out of the five targets, as shown in Fig. 1a, to affirm the role of PLCL1 in RA, model of K/BxN was established by crossing KRN mice with NOD mice. Histopathological analysis (Fig. 1b) demonstrates the model of K/BxN mice was established successfully, increased prominent inflammatory cells and FLS infiltrations. The histology sections of joints were qualitatively assessed for various histopathology features scoring from 0 to 3. Specific Observation items and scores as shown in Table 2. Based on the previous step, we also did an immunohistochemical analysis to demonstrate that PLCL1's expression is higher in synovial tissue of K/BxN mice than in KRN mice. We used Image Pro to detect the integrated optical density (IOD) of PLCL1 and analyzed its value by Prism 8.0 (Fig. 1c). It was found that PLCL1 was significantly increased in the ankle joint of K/BxN mice. OA and RA synovial tissues were also identified by histopathological analysis (Fig. 1d). In the RA synovial tissues compared to the OA synovial tissues, the inflammatory characteristics were more obvious, with clearly increased inflammatory cell infiltration. We used immunohistochemical and WB to analysis the expression of PLCL1 protein was up-regulated remarkably in RA synovial tissues compared with OA, as shown in Fig. 1e, f.

Effect of stimulating RA FLS imitating the inflammatory microenvironment in the joint cavity of RA patients

To imitate the inflammatory microenvironment in the joint cavity of RA patients, we used IL-1β, IL-6, CXCL8, IL-17A, TNF-α, IFN-γ and LPS to stimulate FLS whose working concentrations were 2 ng/ml, 5 ng/ml, 10 ng/ml, 10 ng/ml, 10 ng/ml, 10 ng/ml and 1ug/ml. The level of PLCL1 mRNA were increased under the stimulation of CXCL8, IL-17A, TNF-α and IFN-γ we can see in Fig. 2a. We chose TNF-α to stimulate FLS, using five sequentially increasing concentrations. The expression of PLCL1 protein and other inflammatory cytokines mRNA in FLS was measured by WB and RT-QPCR, respectively (Fig. 2b, c and d). Finally, we examined the expression of PLCL1 protein before and after being stimulated by TNF-α (10 ng/ml) for 48 h with immunofluorescence double staining. The Vimentin was simultaneously stained to indicate that the cells derived from synovial tissues was FLS (Fig. 2e). The result of WB and immunofluorescence both showed that the expression of PLCL1 protein was elevated after the stimulation by TNF-α.

Effect of PLCL1-siRNA inhibited FLS Expressing inflammatory cytokines.

To provide credible evidence that PLCL1 regulates the inflammatory response of RA FLS, we used the siRNA for human PLCL1 specific knockdown of RA FLS gene expression. First, we verified the transfection efficiency of three PLCL1siRNA by RT-QPCR and WB, as Fig. 3a, b can see. We chose SI-2 because its transfection efficiency was up to 50% on average. We divided the cells into four groups, untreated RA FLS, RA FLS stimulated by TNF-α (10 ng/ml) alone for 2 d, RA FLS respectively treated with negative control

(See figure on next page.)

Fig. 1 The expression of PLCL1 was up-regulated in the K/BxN mice and RA FLS. **a** The mRNA level of IL12RB2, PLCL1, CCR2, TCF7 and IQGAP1 was analyzed by RT-QPCR in OA and RA synovial tissue. **b** Typical H&E staining of synovial tissues and their pathology scores in KRN mice and the K/BxN mice (original magnification, × 20). **c** The expression of PLCL1 in synovial tissue of the KRN mice and the K/BxN mice was analyzed by IHC staining. **d** Typical H&E staining of OA and RA synovial tissues (original magnification, × 20). **e** The level of PLCL1 protein in OA and RA synovial tissue was analyzed by IHC staining in human. **f** The level of PLCL1 protein in OA and RA synovial tissue was detected by WB. (Compared with the control group $^{\#}P<0.05$, $^{\#\#}P<0.01$.)

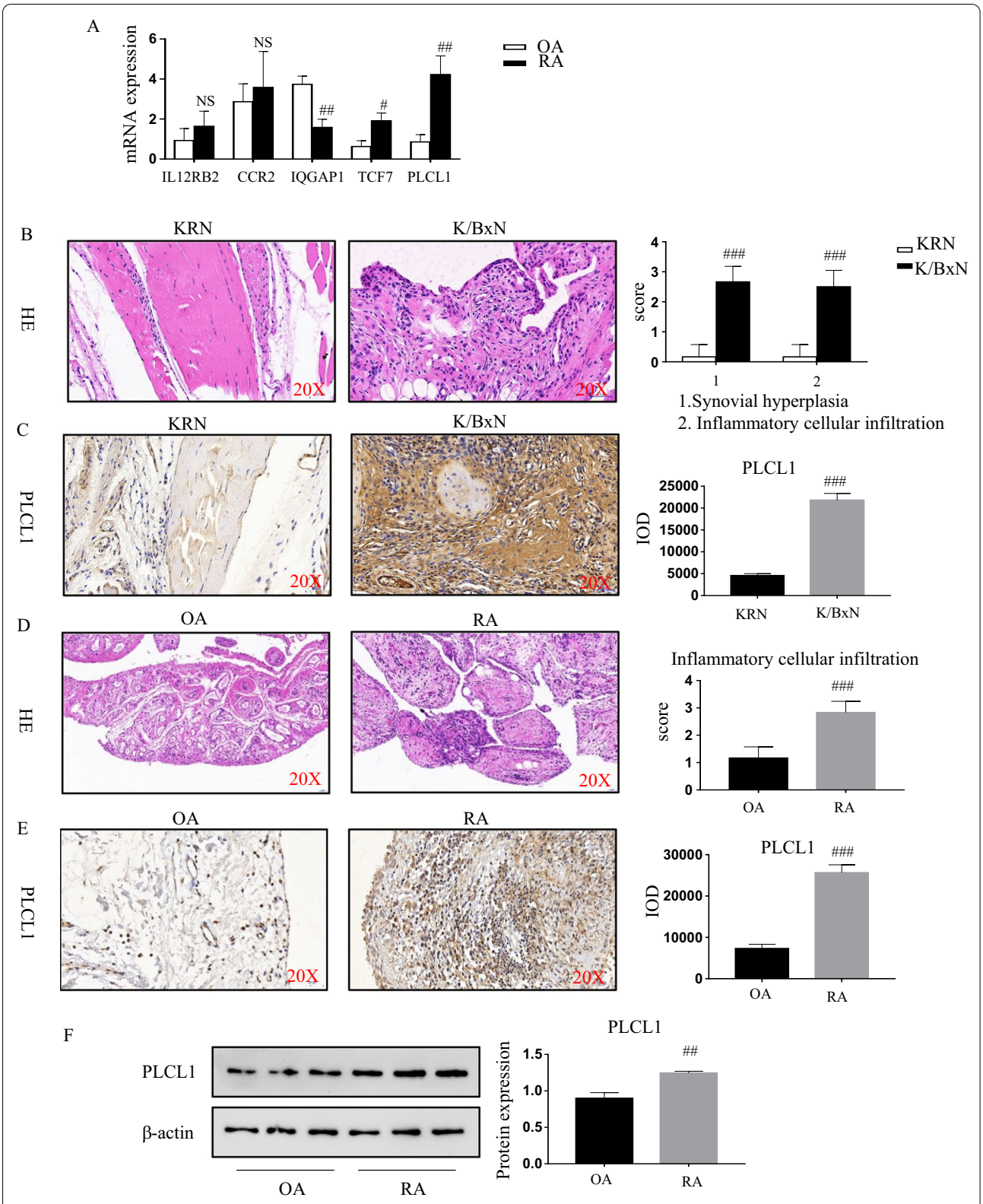


Fig. 1 (See legend on previous page.)

Table 2 The histology sections of joints were qualitatively assessed for various histopathology features scoring from 0 to 3, as follows

Observation	normal	mild	moderate	severe
Synovial hyperplasia	0	1	2	3
Inflammatory cellular infiltration	0	1	2	3

siRNA and PLCL1siRNA, which was stimulated by TNF-α (10 ng/ml) for 2 d following the 6 h of transfection with 100 nM siRNA. The level of PLCL1 protein was decreased observably compared with the cells transfected with negative control siRNA (Fig. 3c). To detect the effect of PLCL1 on FLS, we examined the levels of IL-6, IL-1β and CXCL8 in FLS and supernatants with RT-QPCR and ELISA. In addition, we also analyzed IL-6 and IL-1β in FLS by WB. The results (Fig. 3d, e and f) are in line with our expectation that PLCL1 could promote the expression of inflammatory cytokines in RA FLS.

Effect of PLCL1 overexpression promoted FLS expressing inflammatory cytokines

To further verify the function of PLCL1 in FLS inflammation, we used the overexpression plasmids. The grouping and manipulation of the overexpression experiments were almost identical to the silencing experiments. We divided the cells into four groups, untreated RA FLS, RA FLS stimulated by TNF-α (10 ng/ml) alone for 2 d, RA FLS respectively treated with negative control pcDNA3.1 and PLCL1 pcDNA3.1, which was stimulated by TNF-α (10 ng/ml) for 2 d following the 6 h of transfection with 200 ng pcDNA3.1. As we expected, the expression of PLCL1, IL-6 and IL-1β protein was up-regulated by PLCL1overexpression plasmids as shown in Fig. 4a. We examined the levels of IL-6, IL-1β and CXCL8 in FLS and supernatants with RT-QPCR and ELISA again, as shown in Fig. 4b, c.

PLCL1 plays an important function under the regulation of the NLRP3 inflammasomes

Studies in our laboratory and others have shown IL-1β was positively correlated with NLRP3 inflammasomes. Therefore, in the stimulated FLS, when PLCL1 was silenced, the protein expression of NLRP3 and Cleaved Caspase-1 were decreased as shown in the WB results (Fig. 5a). In contrast, when PLCL1 was overexpressed, the protein expression of NLRP3 and Cleaved Caspase-1 were significantly increased (Fig. 5b). In addition, we used NLRP3 inhibitor INF39 to inhibit the activation of NLRP3. The WB experiment showed that (Fig. 5c) INF39 can effectively inhibit the up regulation of inflammation caused by overexpression PLCL1. So PLCL1 may promote inflammation in RA by regulating the NLRP3 inflammasomes.

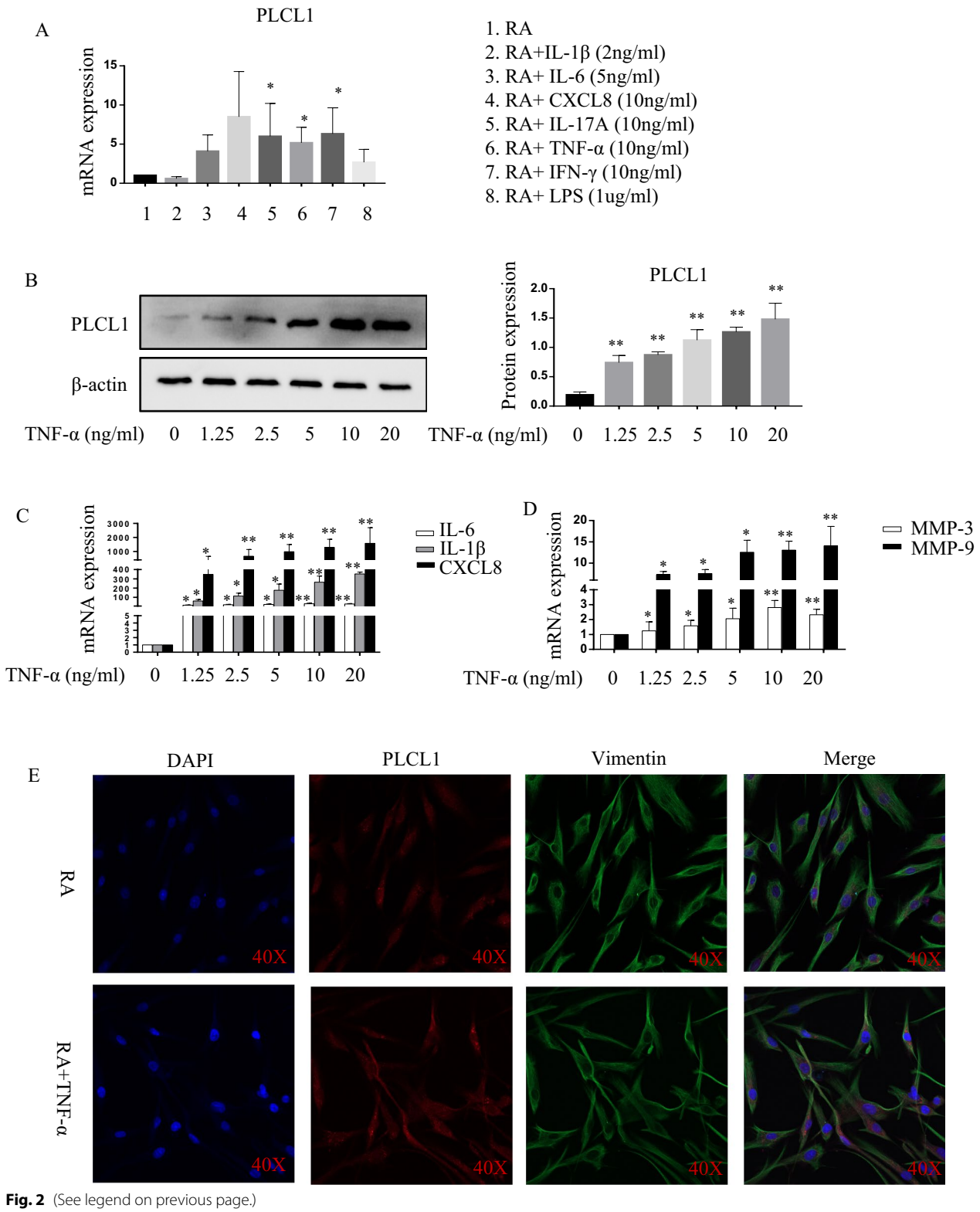
Discussion

In this study, our results showed that PLCL1 was significantly higher in RA and the K/BxN mice the in OA and the KRN mice. Silencing or overexpression of PLCL1 inhibits or intensifies FLS inflammation by regulating NLRP3 inflammasomes. But in fact, we have a small sample size, and we are going to collect more samples to upgrade our experimental data in the future. We choose to stimulate FLS with TNF-α to mimic the inflammatory microenvironment of the joint cavity (Xu et al. [20]; Dong et al. [21]), because TNF-α plays the greatest role in the pathological mechanism of RA (Du et al. [22]). And our previous experimental results have revealed that stimulating FLS with TNF-α (10 ng/ml) could promote inflammatory cytokines production. PLCL1 was positively correlated with the expression of CXCL8, IL-6 and IL-1β in the activated FLS. This is consistent with our expectation that PLCL1 plays a pro-inflammatory role in RA FLS.

PLCL1 promoted the expression of IL-1β. It has been shown that IL-1β is closely associated with the NLRP3 inflammasomes in FLS (Dong et al. [21]). In response to CRT, oligomer of NLRP3 would be formed, which caused the cleavage of caspase-1 and triggered the cleaved IL-1β secretion (Liu et al. [23]). Therefore, we propose the hypothesis that PLCL1 promotes inflammation by regulating the NLRP3 inflammasomes. We used INF39, an exclusive inhibitor to NLRP3 inflammasomes activation, which specifically inhibits NLRP3 activation rather than the NLRC4 or AIM2 inflammasomes (Donget al. [21], Shi et al. [24]). In addition, research have shown that INF39 inhibited NLRP3 inflammasomes activation by reducing

(See figure on next page.)

Fig. 2 Effect of stimulating RA FLS imitating the inflammatory microenvironment in joint cavity of RA patients. **a** The mRNA level of PLCL1 in RA FLS was analyzed by RT-QPCR after being stimulated by IL-17A, IFN-γ, CXCL8, TNF-a, IL-1β, IL-6 and LPS respectively. **b** The protein level of PLCL1 in RA FLS was analyzed by WB after being stimulated by 0, 2.5, 5, 10 and 20 ng/ml TNF-α. **c** The mRNA level of IL-6, CXCL8 and IL-1β in RA FLS was analyzed by RT-QPCR after being stimulated by 0, 2.5, 5, 10 and 20 ng/ml TNF-α. **d** The mRNA level of MMP-3 and MMP-9 was analyzed by RT-QPCR in RA FLS after being stimulated by 0, 2.5, 5, 10 and 20 ng/ml TNF-α. **e** The expression of PLCL1 and Vimentin were analyzed by double immunofuorescence staining analysis in RA FLS before and after being stimulated by 10 ng/ml TNF-α. (Compared with the RA group $^{\#}P<0.05$, $^{\#\#}P<0.01$.)



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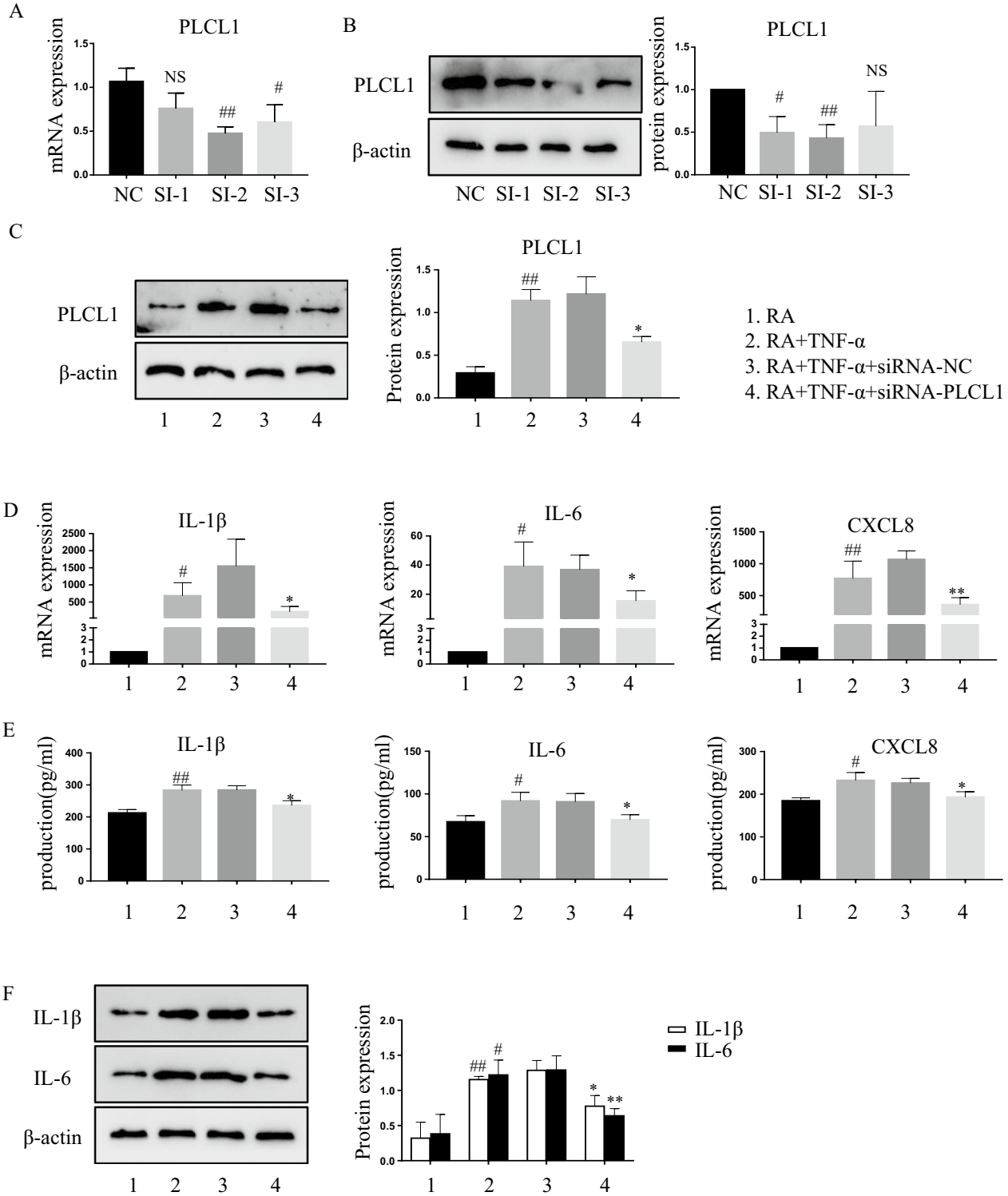

Fig. 3 Effect of PLCL1-siRNA inhibited FLS Expressing inflammatory cytokines. **a**, **b** Detecting transfection efficiency of the three transfected strands in RA FLS by RT-QPCR and WB. **c** The level of PLCL1 protein in 10 ng/ml TNF-α-incubated RA FLS were analyzed by WB, after using PLCL1siRNA. **d** The mRNA level of IL-6, CXCL8 and IL-1β was analyzed by RT-QPCR in TNF-α-incubated in RA FLS after using PLCL1siRNA. **e** ELISA was used to detect the production of IL-6, CXCL8 and IL-1β in TNF-α-incubated RA FLS supernatants after using PLCL1-siRNA. **f** The protein level of IL-6 and IL-1β was analyzed by WB in TNF-α-incubated in RA FLS after using PLCL1-siRNA. (Compared with the RA group $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared with the negative control group $^{*}P<0.05$, $^{**}P<0.01$.)

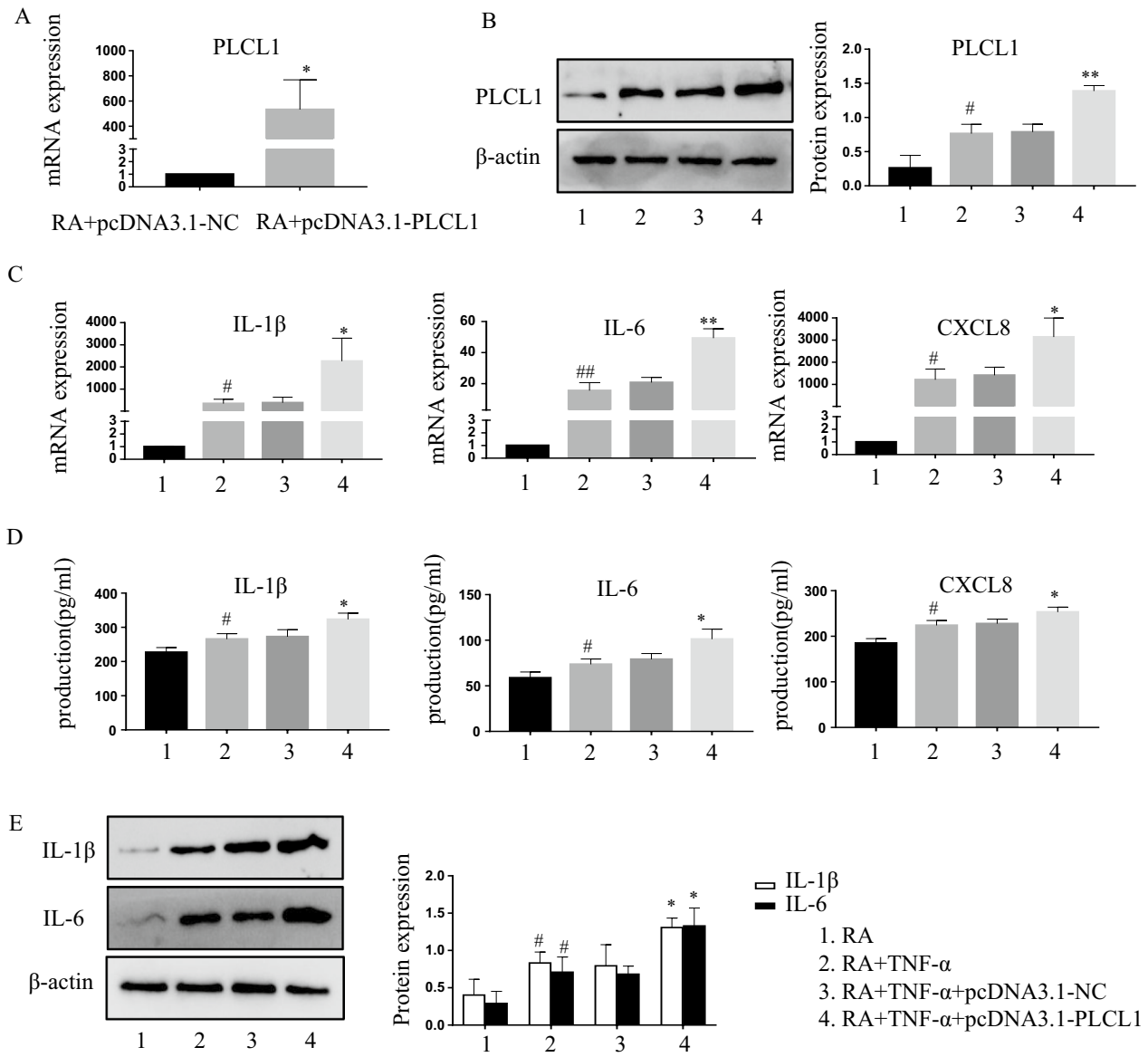

Fig. 4 Effect of PLCL1 overexpression promoted FLS expressing inflammatory cytokines. **a** The overexpression efficiency of PLCL1 were analyzed by RT-QPCR in RA FLS. **b** The protein levels of PLCL1 were analyzed by WB in TNF-α-incubated in RA FLS after using PLCL1 overexpression plasmids. **c** The mRNA levels of IL-6, CXCL8 and IL-1β were analyzed by RT-QPCR in TNF-α-incubated in RA FLS after using PLCL1 overexpression plasmids. **d** ELISA was used to detect the production of IL-6, CXCL8 and IL-1β in TNF-α-incubated RA FLS supernatants after using PLCL1 overexpression plasmids. **e** The protein levels of IL-6 and IL-1β were analyzed by WB in 10 ng/ml TNF-α-incubated in RA FLS after using PLCL1 overexpression plasmids. (Compared with the RA group $^{\#}$ $P<0.05$, $^{\#\#}$ $P<0.01$; compared with the negative control group * $P<0.05$, ** $P<0.01$.)

NLRP3 expression, and this phenomenon was also found in our study after INF39 use (Shi et al. [24]). In this study, INF39 effectively inhibited the proinflammatory effects caused by overexpression of PLCL1.

Although the nosogenesis of RA is unclear, the role of inflammatory cytokines in RA pathogenesis is undeniable (Mateen et al. [8]; Albeltagy et al. [25]). In RA, activated FLS express chemokine to recruit B cells, T cells, macrophages, natural killer cells, and neutrophils into the joint cavities to "respond" to each other (Kumar et al. [26], [5]. Among them, inflammatory cytokines are the key substances in the mutual "response" between cells (Li et al. [27]). The TNF-α, IL-1β, CXCL8 and IL-6 involved in this study are several of the most classical inflammatory cytokines in RA (Mateen et al. [8]). Their levels were all significantly increased in the joint synovium of

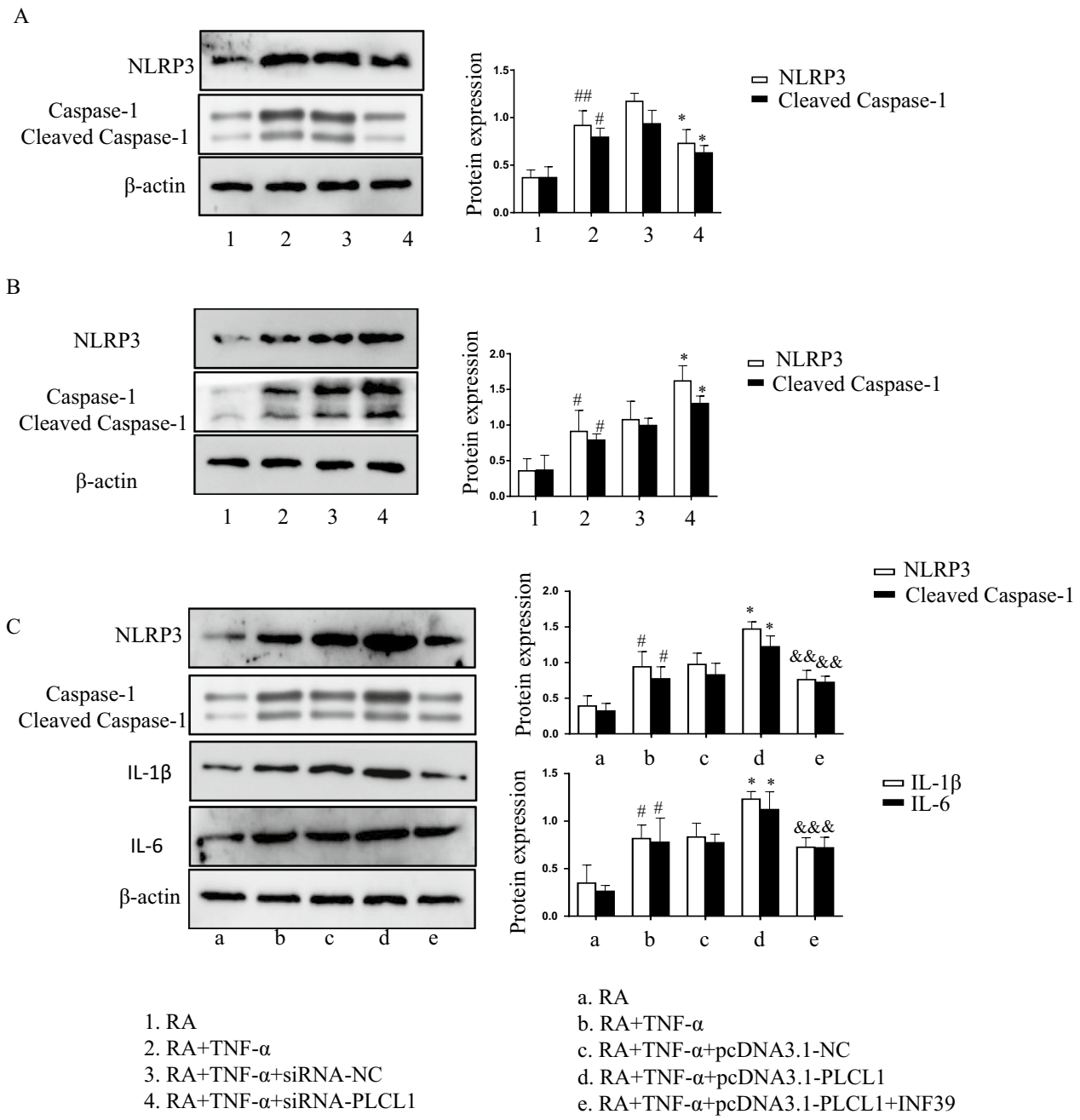

Fig. 5 PLCL1 plays an important function under the regulation of the NLRP3 inflammasomes. **a** The level of NLRP3 protein and Caspase-1/Cleaved Caspase-1 protein in 10 ng/ml TNF-α-incubated RA FLS were analyzed by WB after using PLCL1siRNA. **b** The protein level of NLRP3 and Caspase-1/Cleaved Caspase-1 were analyzed by WB in 10 ng/ml TNF-α-incubated RA FLS after using PLCL1 overexpression plasmids. **c** WB was used to detect NLRP3, Caspase-1/Cleaved Caspase-1, IL-6 and IL-1β protein levels in 10 ng/ml TNF-α-incubated RA FLS after using PLCL1 overexpression plasmids and NLRP3 inhibitor INF39. (Compared with the RA group $^{\#}P<0.05$, $^{\#\#}P<0.01$; compared with the negative control group $^{*}P<0.05$, $^{**}P<0.01$; compared with the PLCL1 overexpression group $^{\&}P<0.05$, $^{\&\&}P<0.01$.)

RA patients.IL-6 showed synergy with IL-1β and TNF-α to produce vascular endothelial growth factors (VEGF), which act on vascular endothelial cells to increase their permeability, eventually leads to increased swelling by the accumulation of joint effusion [28]. CXCL8 serving as a chemokine in RA trend and induce neutrophil to release a cartilage-degrading enzyme (An et al. [29]).

Conclusions

The finding in this study suggested that PLCL1 might serve as a critical role during FLS activation and be closely related to the NLRP3 inflammasomes. Silencing of PLCL1 inhibiting inflammation of FLS is a more efficient and qualified view, suggesting the possibility of PLCL1 as a therapeutic target for RA. Further studies are needed to elucidate whether PLCL1 can serve as a diagnostic marker and prognostic indicator of RA.

Acknowledgements
Thanks to Prof. Christophe Benoist and Prof. Diane Mathis of Harvard Medical School for providing KRN mice for free. We also thank Dr. Chengbiao Ding, Rehabilitation Department of The Second Affiliated Hospital of Anhui Medical University, for providing synovial tissue of RA and OA.

Author contributions
SL conceived and design the study. All authors have contributed to the development of the manuscript. All authors read and approved the final manuscript.

Funding
This research was funded by Anhui Provincial Science and Technology Major Project (No. 8212929035); the University Synergy Innovation Program of Anhui Province (GXXT-2019–045, GXXT-2020–063, GXXT-2020–025); National Natural Science Foundation of China (No. 82002269); China Postdoctoral Science Foundation (No. 2020M671839); Postdoctoral Science Foundation from Anhui Medical University (No. BSH201902).

Availability of data and materials
Available under request.

Declarations

Ethics approval and consent to participate
This study plan was aproved by the institutional Ethics committee of Anhui Medical University (No.20200872) and the Animals Use and Care Committee (No.20200863). In this case we collected patients' synovial tissue according to the guidelines of the institution. We also strictly abide by the Administration of Experimental Animal regulations issued by China's State Science and Technology Commission.

Consent for publication
Not applicable.

Competing interests
None of the authors have a conflict of interest.

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Received: 13 December 2021 Accepted: 8 June 2022
Published online: 11 July 2022

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