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Intervening the up-regulated SLC7A5 could mitigate the inflammatory mediator by mTOR-P70S6K signal in rheumatoid arthritis synoviocytes.

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Abstract

Objective The increased bioenergetic and biosynthetic demands of sustained inflammation and changes to nutrient and oxygen availability are found in rheumatoid arthritis (RA). This study aimed to observe the effects of SLC7A5 (amino acid transporter) on synoviocytes of RA patients and pinpoint the underlying molecular mechanisms.

Methods Synovial tissues were collected from OA and RA patients. Fibroblast-like synoviocytes (FLS) were isolated from synovial tissues from RA patients. SLC7A5 expression was evaluated by using RT-qPCR, immunofluorescence and Western blotting. Matrix metalloproteinases (MMPs) expression was evaluated by using RT-qPCR and Western blotting. RNAi and antibody blocking treatments were used to knockdown the expression of SLC7A5 or block its transporting function.

Results The SLC7A5 expression was significantly upregulated in the FLS from RA patients compared with that in FLS from OA patients. Cytokine IL-1β played a crucial role in up-regulating SLC7A5 expression via NF-κB pathway in FLS. Intervening SLC7A5 expression with RNAi or blocking SLC7A5 function by monoclonal antibody could ameliorate the MMP3 and MMP13 protein expression. Furthermore, up regulation of SLC7A5 enhanced mTOR-P70SK6 signaling activation which could promote the protein translation of MMP3 and MMP13 in RA FLS.

Conclusion SLC7A5 up-regulation could be induced by activated NF-κB pathway, further resulted in an enhanced mTOR-P70S6K activity and the protein expression of MMP3 and MMP13 in FLS from RA patients.

Background

Rheumatoid arthritis (RA) is a chronic autoimmune joint disease with the global prevalence of RA was 0.24% [1]. This disease characteristically involves the synovial hyperplasia and progressive destruction in small joints. The detailed pathogenesis of RA is complex and many cell types, such as T cells, B cells, macrophages and fibroblast-like synoviocytes (FLS) participate in the process. Among all these cells, FLS may play the key role in RA development by producing the inflammatory cytokines and the proteases which perpetuate inflammation and destroy cartilage, respectively [2]. Moreover, it has been described that the FLS in RA manifest distinguished phenotypes such as tumor-like hyperplasia and aggression [3]. The FLS aggressively invade extracellular matrix (ECM) and further exacerbate joint damage by secreting proinflammatory cytokines, chemokines and matrix metalloproteinases (MMPs) [4, 5]. Meanwhile, these quickly proliferated FLS need high energy demand, which is well associated with high-level transportation and consumption of glucose and amino acids.

Solute carrier family 7 member 5 (SLC7A5), alias L-type amino acid transporter (LAT1) [6], is a sodium-independent transporter with high-affinity to amino acids. SLC7A5 working together with SLC3A2 involves in cellular uptake of the large neutral amino acids such as phenylalanine, tyrosine, leucine, and tryptophan [7]. The SLC7A5 is mainly distributed in placenta, testis, bone marrow and brain by using

Northern blotting, whereas the expression of SLC3A2 is more ubiquitous [8]. Global knockout of Slc7a5 could result in an embryonic lethal phenotype in mice, partially due to a deleterious effect upon Slc7a5 transporting function during post-implantation embryonic development [9, 10]. The conditional knockout animal of Slc7a5 shows that Slc7a5 works as a checkpoint in T cell activation via mTORC1 complex [11]. Meanwhile, the hypoxia-inducible factor 2α binds to the SLC7A5 proximal promoter and drives its transcription in WT8 cell line [12]. And in the inflamed RA joints, the hypoxic condition becomes increasingly severe due to the metabolically consumption in those active cells [13]. Recently, an mRNA expression profiling study using microarray showed that the SLC7A5 is overexpressed in RA synovial tissue [14].

According to the above-mentioned clues, there might be the deep implication for SLC7A5 molecule during RA pathogenesis, however, its possible role and actual function in the inflamed FLS remains poorly understood. Hence, we explore the role of SLC7A5 and pinpoint underlying molecular mechanism in FLS of RA patients.

Methods

Patients' samples

All the synovial tissues and FLS were derived from patients with RA and OA during knee joint replacement surgery (Department of Joint Surgery, Honghui Hospital, Xi'an Jiaotong University, China). All the patients' data were summarized in the supplemental material Table 1. All the patients who are involved in this study were signed the patient consent. This study was approved by the Medical Ethics Committee of Xi'an Jiaotong University (No. 2016-261 and No.2017-666).

Table 1

Patient characteristics

Clinical Data	RA	OA
Number of patients	24	24
Sex		
Female	17	19
Male	7	5
Age[#]	56.71±1.673	66.38±1.396
CRP[#] (mg/L)	29.30±3.615	4.25±1.608
RF[#] (IU/mL)	92.57±15.07	6.846±0.9816
ESR[#] (mm/h)	64.83±7.18	15.79±3.774

CRP, C-reactive protein, RF, rheumatoid factor, ESR, erythrocyte sedimentation rate

[#] Mean ± SEM

Histology and immunofluorescence

The histological assay was performed on the synovial tissue paraffin sections from OA and RA patients after staining with hematoxylin and eosin (H&E). The synovial tissue paraffin sections (6 μm thick) were used for immunofluorescent staining. A mixture of primary antibodies included mouse monoclonal antibody against SLC7A5 (1:100, Santa Cruz, sc-374232) and rabbit polyclonal antibody to Vimentin (1:100, Bioss, bs-23064R) were applied on the sections overnight. The next morning the secondary antibodies, FITC AffiniPure goat anti-rabbit IgG (H+L) (1:400, Earthox, E031210-01) and Cy3 AffiniPure goat anti-rabbit IgG (H+L) (1:400, Earthox, E031620-01) were applied on the same sections after washing. The DAPI was used to detect the nucleus (1:100000, Sigma-Aldrich, D9542). Immunofluorescent staining was carried out followed the described procedure with slight modification [15]. The immunofluorescent images were captured with a fluorescence microscope (Olympus, Japan) and merged by Image J software.

Cytokines and inhibitor treatment

To explore which cytokine could induce SLC7A5 expression in FLS, various cytokines including IL-1β (20 ng/mL), TNF-α (20 ng/mL), IFN-γ (20 ng/mL), IL-6 (20 ng/mL) and IL-17A (20 ng/mL) (Genscript, China) were used to treat the cells for 24 hours. The total protein was collected for the further Western blotting assay.

To verify the upstream of SLC7A5 expression, the JNK inhibitor SP600125 (10 μM, Selleckchem, s1460) and NF-κB inhibitor $BAY_{11\text{-}7085}$ (10 μM, Selleckchem, s7352) were used to treat the cells for 4 hours before 20 ng/mL IL-1β was used to stimulate the cells for 24 hours. The total mRNA and protein were collected for further RT-qPCR and Western blotting assay.

Blocking assay of SLC7A5

SLC7A5 antibody (20 μg/mL, a mouse anti- SLC7A5 monoclonal antibody, IgG_1, Santa cruz, USA) was administrated to FLS. The detailed procedure was described in our previous paper[16]. Briefly, FLS were seeded in 12 well plates at a density of 4×10^4/mL and incubated with SLC7A5 antibody or isotype-matched IgG_1 (CST, #5415, USA) for 24 hours, and then treated with IL-1β for 18 hours. Afterward, the cells were collected for detecting the mRNA and protein level of MMP3 and MMP13 respectively.

Western blotting

Total protein lysates from synovial tissues and cells were extracted by using the RIPA solution (Beyotime, China) with a cocktail of protease and phosphatase inhibitors (Roche). The final protein concentration of each sample was determined by a BCA kit (Thermo Scientific, USA). Total proteins of 20 μg from cell lysates were separated by 6% or 8% SDS-PAGE gels according to standard procedures with the Bio-Rad system. The primary antibodies were incubated at 4°C overnight. The primary antibodies list were in supplemental table S5. The signal was further detected by using the secondary antibody of goat anti-rabbit IgG conjugated with HRP or goat anti-mouse IgG conjugated with HRP (0.4 μg/ml, Abcam, USA). Signal intensity was determined by Supersignal® West Pico Kit (Thermo Scientific). All the density of the

bands was measured by Image J software and normalized to β-actin. Final data are expressed by showing one representative image and quantitative result of samples from various patients.

RNA isolation and RT-qPCR

Total RNA from synovial tissues and cells was isolated with TRI Reagent™ solution (Thermo Scientific, USA), and cDNA was synthesized by First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. RT-qPCR was performed by using iQ5 optical system software (Bio-Rad Laboratories, USA) with Fast Start Universal SYBR Green Master (ROX) (Roche, USA) for mRNA quantitation of all referred genes. The information of all the primers, products and annealing temperatures is depicted in Table S1. Gene expression analyses were performed against *GAPDH* expression and calculated by using the $2^{-\Delta\Delta Ct}$ method.

RNAi

Both small interfering RNA (siRNA) targeting SLC7A5 (si1:5'- CATTATACAGCGGCCTCTTT-3', si2: 5'-TAGATCCCAACTTCTCATTT-3') and Negative control (NC, 5'-GCGACGAUCUGCCUAAGAUTT-3') were purchased from Oligobio (Beijing, China). Cells were transfected with either SLC7A5 siRNA or NC siRNA at 75 nmol/L using Lipofectamine™ 2000 Transfection Reagent (Thermo Scientific, USA) according to the manufacturer's guidelines. The cells were transfected for 48 hours to validate the RNAi efficiency by Western blotting. The cells were firstly transfected 24 hours with siRNAs respectively and then treated with IL-1β to detect the effects and the signal pathways.

Cytokine profiling assay

RA FLS (seeded at 2×10^5 cells/mL overnight) in 6 well plate were transfected with siRNA, and 4h later the medium was changed into DMEM containing 0.2%FBS in a total of 2 ml and incubated for 48 hours. Such conditioned supernatant was collected, centrifuged at 2000 rpm for 10 min at 4 ℃, and aliquots were stored at -80 ℃ before use.

Secreted cytokine expression in siRNA treated RA FLS supernatants were detected using RayBio® C-Series human cytokine antibody array (AAH-CYT-5). Membrane based dot ELISA coated with 80 human cytokines (listed in the supplemental material Table S2) were further incubated with RA FLS supernatants pooled from 4 donors either transfected with *SLC7A5* siRNA or the negative control molecules for 48h. The detection and analysis of this cytokine array were performed by RayBiotech Company according to the manufacturer's instruction. Dot immunoblot signal from array membrane incubated with supernatant sample paired with negative control was captured and the raw intensity was showed in the supplemental material Table S3.

Statistics

Data were expressed in the mean ± standard error of mean and SPSS software was used for analysis. One-way ANOVA among groups, and the Student's t-test or Mann-Whitney-Wilcoxon test was employed to

analyze the significant differences between the two groups according to the distribution of the data (normal distribution was validated using Shapiro-Wilk test). *p* less than 0.05 was considered statistically significant.

Results

SLC7A5 expression is up-regulated in fibroblast-like synoviocytes from RA patients.

To evaluate the contribution of SLC7A5 during RA pathogenesis, we collected the synovial tissues from RA and OA patients. The histology results showed that the synovial tissues from RA patients were heavily proliferated and infiltrated with inflammatory cells, compared with those of OA patient (Fig. 1A). The mRNA expression of SLC7A5 was significantly up-regulated in synovial tissues from RA patients as compared to that from OA patients (Fig. 1B). In addition, we performed the correlation analysis between the SLC7A5 mRNA expression and serum RF as well as CRP. The results showed a significantly positive correlation between not only SLC7A5 mRNA level and RF (Fig. 1C), but also SLC7A5 mRNA level and CRP (Fig. 1D). And then the protein level of SLC7A5 was examined. Clearly, the protein expression of SLC7A5 was also up-regulated significantly in synovial tissues of RA patients compared with OA patients (Fig. 1E). The immunofluorescence staining on the consecutively followed slide of synovial tissue showed in Fig. 1F. SLC7A5 was overexpressed and co-localized in vimentin-positive cells (FLS) in synovial tissues from RA patients.

The up-regulation of SLC7A5 expression is mediated by IL-1β via an NF-κB pathway

To investigate which molecule should be responsible for the up-regulated SLC7A5 in FLS, we focused on proinflammatory cytokines, one of the most important contributors in RA. Interestingly, we found that both IL-1β and IL-6 could significantly up-regulate SLC7A5 protein expression respectively (Fig. 2A). And the expression of SLC7A5 in the IL-1β treatment group showed a little higher than the IL-6 treatment group. From Fig. 2B, IL-1β could activate both the JNK and NF-κB signaling pathways in FLS by either phosphorylating JNK or promoting IκB degradation. To make sure which pathway was related to up-regulating SLC7A5 expression, the inhibitor of these two signal pathways, SP600125 (JNK signaling inhibitor) and $Bay_{11-7085}$ (NF-κB signaling inhibitor), were used respectively to treat the FLS stimulated by IL-1β. The data demonstrated that the activated NF-κB signaling rather than JNK pathway played a crucial role during SLC7A5 up-regulation (Fig. 2C-D).

Up-regulated SLC7A5 enhances MMP3 and MMP13 protein expression in FLS

To figure out the function of SLC7A5, an amino acid transport, in activated FLS, the monoclonal antibody of SLC7A5 was used as a blocker. The results showed that there was no change in the mRNA level of MMP3 and MMP13 after blocking the SLC7A5 (Fig. 3A), but the protein expression of MMP3 and MMP13

were decreased after SLC7A5 blocked by its own antibody (Fig. 3B-C), which indicated that the suppression of MMP3 and MMP13 expression happened only at the protein level rather than mRNA level.

The RNAi was also performed to confirm the SLC7A5 function in RA FLS. The result of SLC7A5 protein expression showed that SLC7A5 siRNA No.2 had a significant effect in down-regulating the expression of SLC7A5 in FLS after transfected with siRNA for 48 hours (Fig. 3D). Meanwhile, the mRNA of SLC7A5 was also decreased after transfected with siRNA (Fig. 3E). And the result also showed that although the siRNA down-regulated SLC7A5 mRNA expression successfully (Fig. 3F), there was no change in the mRNA level of MMP3 and MMP13 (Fig. 3G-H). Then we detected the protein expression of MMP3 and MMP13 again in the down-regulated SLC7A5 FLS and its negative control cells. The results showed that both the protein level of MMP3 and MMP13 had changed after knocking down the SLC7A5 expression (Fig. 3I-J). These results indicated the SLC7A5 played an important role in regulating the MMP3 and MMP13 protein expression in RA FLS.

A total of 80 human cytokines were detected (Fig. 4A) in conditioned media of RA FLSs after transfected with SLC7A5 siRNA for 48 h (Fig. 3E). Semi-quantitative data showed that the expression fold change of increased IL-10, PARC, PLGF, TGFβ_2, TGFβ_3, TIMP1 and decreased PDGF-BB were beyond ± 1.5 (plotted in Fig. 4B, and data were described in supplemental material Table S2, S3, and Supplementary Fig.S). KEGG pathway analysis predicted that (supplemental material Table S4) multiple pathways were significantly related to this altered cytokine profiling after SLC7A5 knockdown. Among them, it is in particular interest that these pathways also included inflammatory bowel disease (IBD) and rheumatoid arthritis (Fig. 4C).

Up-regulated SLC7A5 activates mTOR-P70S6K signal and enhances MMP3 and MMP13 expression in FLS

To reveal the mechanism of how SLC7A5 regulates the protein level of MMP3 and MMP13, the amino acid sensor mTOR and its substrate were detected in synovia tissues and inflamed FLS. As the results Fig. 5A-C showed, compared with OA synovial tissues, the expression of P70S6K and p-mTOR were significantly up-regulated in RA synovial tissues. When FLS were treated with IL-1β, the same results were obtained. The expression of SLC7A5 had significantly up-regulated as well as the phosphorylation of P70S6K in FLS treated with IL-1β (Fig. 5D-F). To confirm these results associated with SLC7A5 function in amino acid sensor activation and translation signal pathway, SLC7A5 was knocked down by RNAi in FLS. Figure 6G-H showed that the phosphorylation of mTOR and P70S6K could be intervened significantly after SLC7A5 knocked down. This result suggested that the overexpressed SLC7A5 in FLS from RA patients had a crucial role in the activation of mTOR and p70S6K and further regulation of the protein translation pathway activation.

Discussion

In the present study, we demonstrated that the highly expressed SLC7A5 in the synoviocytes is induced by local IL-1β via NF-κB signal activation. Overexpressed SLC7A5 enhances the activation of mTOR-P70S6K

signal and promoted the protein expression of MMP3 and MMP13 in FLS.

Studies regarding the extent of the metabolic changes and the types of metabolites could provide us suitable biomarkers for RA diagnosis. Mounting evidence supports that the metabolic condition in RA patients is different from that in other arthritis diseases [17]. Multiple amino acids such as glycine, leucine, serine, tyrosine, isoleucine, and proline were found in RA patients synovial fluid [18]. However, only a few studies were focused on amino acid transport in RA pathogenesis. The metabolic change involved in joint inflammation is very complicated, and many interactions may take place, leading to a complex communication network between different cell types. Therefore, more knowledge is needed to unveil the critical interactions between amino acid transporter and FLS function in the arthritis process. In this study, we demonstrated that an amino acid transporter, SLC7A5, was up-regulated in FLS of RA patient, which enhanced the expression of MMPs in protein level, and played a critical role in maintaining the FLS infiltration to destroy the adjacent cartilage and bone. Therefore, blocking the SLC7A5 function may slow down the FLS infiltration in the RA process.

To further understand the mechanism of how SLC7A5 was up-regulated in FLS, the FLS inflammatory microenvironment was studied in the present research. In RA synovial fluid, there are a lot of inflammatory mediators secreted by immune cells. IFN-γ and TNF-α could be secreted by activated T cells [19]. IL-17 was produced by Th17 and mast cells [20]. Activated macrophages were discovered to secret other cytokines include IL-1β, IL-6, TNF-α [21]. These inflammatory cytokines might accelerate the process of matrix degradation by FLS. Hence we used a series of cytokines to stimulate the FLS and detected the SLC7A5 expression. According to our data, we found that IL-1β could up-regulate SLC7A5 expression via NF-κB activation. This result suggested NF-κB activation might play a key role in SLC7A5 expression. Another study showed that LPS could up-regulate SLC7A5 expression in RA monocytes [22]. More interestingly, hypoxia is an important micro-environmental characteristic of RA [23]. HIF-2α, one of hypoxia transcriptional factors, could modulate the expression of mediators that are involved in cellular infiltrate in the synovial tissue, cartilage destruction, and bone erosion. This transcriptional factor was also found binding to the Slc7a5 promoter and increases Slc7a5 expression in normal liver and lung tissues [12]. All these evidence suggested that the up-regulated SLC7A5 not only modulated by NF-κB activation but also might be regulated by different transcriptional factors in RA FLS.

To investigate the function of overexpressed SLC7A5 in RA, we focused on its amino acid transportability. Our data support the hypothesis that amino acid transport could manipulate the FLS function by showing that blocked SLC7A5 function via its antibody or knocked-down the SLC7A5 expression via RNAi. The blocking SLC7A5 transport function study in FLS showed that the protein expression of MMP3 and MMP13 were significantly suppressed. And these two proteinases MMP3 and MMP13 mediated aggrecan and collagen cleavage and degradation in cartilage [24]. Interestingly, using the amino acid transport inhibitor 2-(methylamino) isobutyric acid to treat the arthritis animals could attenuate the severity of arthritis process [25], suggesting that the amino acids and their transports might be a critical character in RA process. In fact, the expression level of MMP3 and MMP13 increases considerably in arthritic synovium and cartilage, resulting in the aberrant destruction of cartilage tissues [26, 27]. So our

results suggest that the enhanced MMP3 and MMP13 protein expression via SLC7A5 might accelerate the collagen degradation and cartilage surface erosion. As we mentioned previously, FLS display some phenotypes like tumor cells and exhibit infiltration behavior to cartilage. Moreover, many SLC7A5 related tumor studies also suggested that SLC7A5 played a critical role in tumor migration and invasion. In the cholangiocarcinoma cells, down-regulation of SLC7A5 expression could decrease cell migration and invasion [28]. SLC7A5 regulated by miR-126-3p exhibits a strong implication relative to cellular migration and metastasis in thyroid cancer cell [29]. All these data support that the SLC7A5 plays a critical role in regulating cell infiltration and invasion and indicated that the overexpressed SLC7A5 might participate in FLS infiltration by enhancing the expression of MMPs.

Our results showed that blocking SLC7A5 by its antibody suppressed only the protein expression of MMP3 and MMP13, while had no effects on their mRNA expression, suggesting that blocking SLC7A5 function might modulate the amino acid sensor. mTOR is a central nutrient sensor that signals protein translation. And mTOR complex (mTORC) has been shown sensitive to the intracellular levels of certain amino acid [30]. Our data showed that knocked-down the expression of SLC7A5 in FLS could decrease the protein level of MMP3 and MMP13 via suppressing the phosphorylation of mTOR and P70S6K significantly. This might influence the amino acid transported by SLC7A5 in FLS. SLC7A5 could mediate amino acid flux and activate mTORC1 in tumor cells and immune cells as well [12, 22]. In SLC7A5 knock-out cancer cell lines, the phosphorylation level of P70S6K decreased, and cell proliferation was compromised [31]. And intracellular amino acids could induce phosphorylated mTOR to activate P70S6K [32]. P70S6K plays important roles in cell growth, proliferation and differentiation by regulating cell cycle progression ribosome biogenesis [33, 34]. It phosphorylated multiple components of the translational machinery and related regulators and increased translation[35]. Hence in RA FLS, overexpressed SLC7A5 promoted phosphorylation of mTOR, which activated the downstream effector P70S6K and enhanced MMP3 and MMP13 protein translation. Additionally, the upstream of mTOR signal, Akt activation could also induce MMP3 and MMP13 expression in microglia [36]. What's more, we found that SLC7A5 siRNA could cause increased IL-10, TIMP1 and decreased PDGF-BB protein production in RA FLS supernatant. Previous literature reported that these cytokines, like IL-10 works as an anti-inflammatory cytokine, inhibits VEGF [37], decrease the inflammatory responses [38]. The increased Timp1 could ameliorate cartilage destruction in collagen-induced arthritis in rats [39]. All these findings are accord with our hypothesis that the amino acid transport SLC7A5 took part in invasion via regulating the protein expression of MMP3 and MMP13 in RA FLS. Down-regulated or blocking SLC7A5 in FLS could serve as an anti-inflammatory target for potential arthritis therapy.

Conclusion

Our current study highlights the dominant function of SLC7A5 in FLS from RA patients. FLS from RA patients are regulated by IL-1β through the NF-κB pathway to express a higher level of SLC7A5. Block the SLC7A5 function via its antibody or RNAi, could inhibit the MMP3 and MMP13 expression in FLS. And overexpressed SLC7A5 enhances the protein production of MMP and MMP13 mediated by mTOR -

P70S6K-translation pathway. The findings provide new insights into the pathogenesis of RA and may pave a special way to intervene the disease.

Abbreviations

RA: Rheumatoid arthritis, FLS: fibroblast-like synoviocytes, MMP: matrix metalloproteinases, SLC7A5: Solute carrier family 7 member 5, CRP: C-reactive protein, RF: rheumatoid factor, ESR: erythrocyte sedimentation rate.

Declarations

Fundings

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Ethics Approval

This study was approved by the Medical Ethics Committee of Xi'an Jiaotong University (No. 2016-261 and No.2017-666).

Consent for publication

There are no details on individuals reported within the manuscript.

Availability of data and supporting materials section

We would like to share our data and materials in the supplementary material.

Contributors

Jing Xu, Congshan Jiang and Shemin Lu conceived, designed and applied the research, analyzed data and wrote the paper. Yongsong Cai, Jiawen Xu, Ke Xu and Peng Xu provided the synovial tissues and persuaded patients to donor their tissue and share their medical information. Jing Xu, Congshan Jiang, Yuanxu Guo, Xipeng Wang, Jiaxiang Zhang, Wenhua Zhu, Si Wang, Fujun Zhang, Manman Geng, Yan

Han, Qilan Ning, and Liesu Meng contributed to acquisition and/or analysis of data. All the authors approved the final version of the paper.

Competing Interests

The authors declare that they have no competing interests.

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Figures

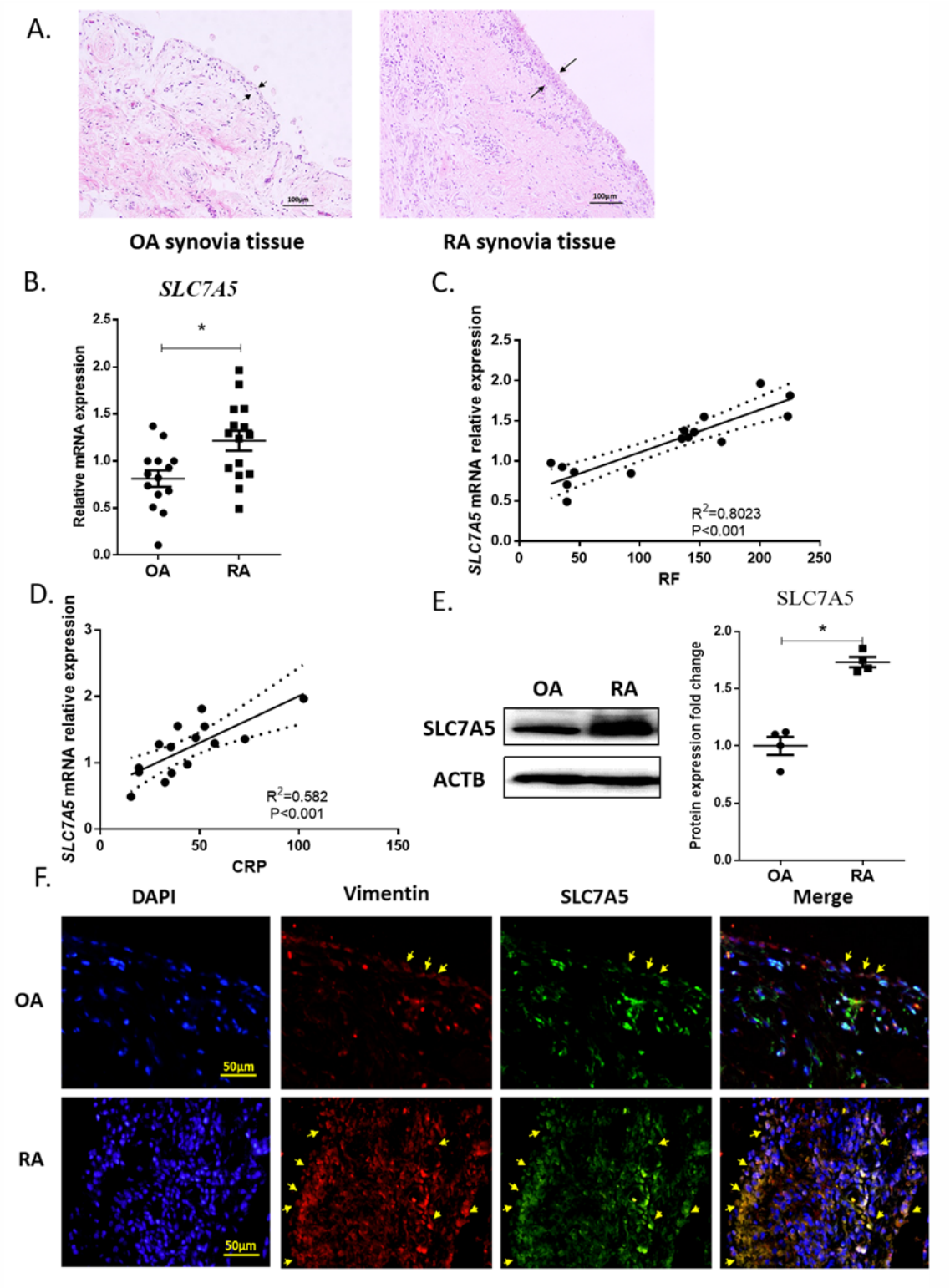

Figure 1

SLC7A5 was up-regulated in fibroblast-like synoviocytes of RA patients. A. Hematoxylin and eosin (HE) staining of the synovial tissue from RA and OA patients. B. The mRNA expression of SLC7A5 in synovial tissue from OA and RA patients was detected by RT-qPCR (RA n=15, OA n=14). C-D. The mRNA expression of SLC7A5 was positively correlated with RF (C) and CRP (D) from RA patients (n=15). E. The protein expression of SLC7A5 in synovial tissue from OA and RA patients detected by Western blotting. And the

density of SLC7A5 immune-reactive bands was analyzed by using ACTB expression as a loading control (RA n=4, OA n=4). F. Representative immunofluorescence staining for SLC7A5 (green) and Vimentin (red) in synovial tissue from OA and RA patients (RA n=3, OA n=3). The slide used for IF stain was consecutively followed slide stained with H&E. The picture showed in Fig.1F was the enlarging arrow area pointed out in Fig.1A. (*: p< 0.05)

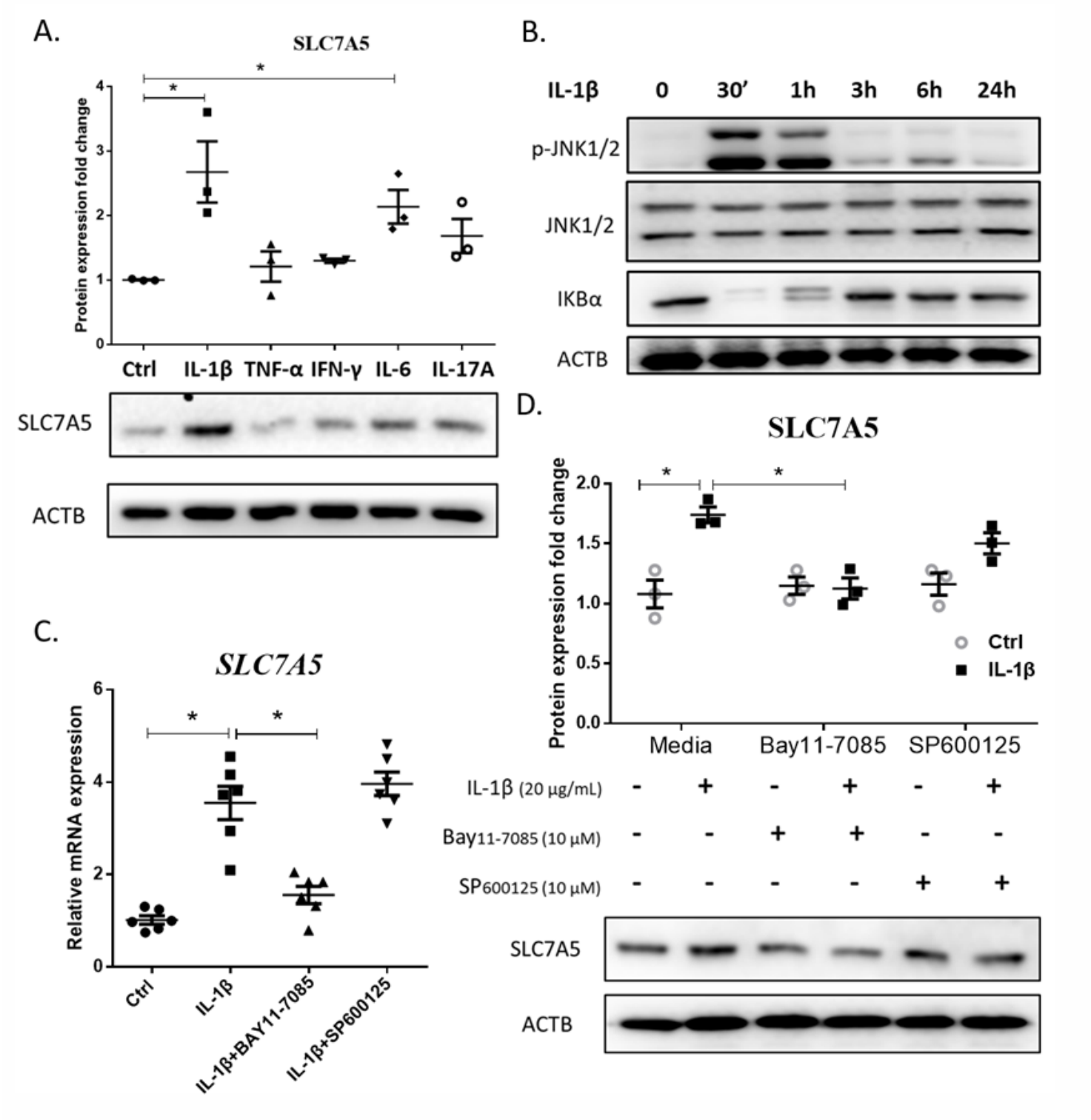

Figure 2

IL-1β played a crucial role in up-regulating SLC7A5 expression via NF-κB in fibroblast-like synoviocytes (FLS). A. The protein expression of SLC7A5 in FLS from RA patients treated with different cytokines (IL-1β 20ng/mL, TNF-α 10ng/mL, IFN-γ 20ng/mL, IL-6 20ng/mL and IL-17A 10ng/mL) for 24 hours was detected by Western blotting. The density of SLC7A5 immune-reactive bands was analyzed by using ACTB expression as a loading control (n=4). B. The representative active signal pathways in FLS from RA patients were detected by Western blotting. The FLS were treated with 20ng/mL IL-1β, and the protein was collected at different time points. C. The mRNA expression of SLC7A5 was changed in FLS from RA patients after stimulated with different inhibitors. The FLS were firstly treated with NF-κB inhibitor Bay11-7085 (10μM) or JNK inhibitor SP600125 (10μM) for 4 hours and then treated with 20ng/mL IL-1β for 24 hours. The mRNA expression was detected by RT-qPCR (n=6). D. The protein expression of SLC7A5 in FLS from RA patients was changed in FLS from RA patients after stimulated with different inhibitors. The cells were firstly treated with NF-κB inhibitor Bay11-7085 (10μM) or JNK inhibitor SP600125 (10μM) for 4 hours and then treated with 20ng/mL IL-1β for 24 hours. The protein expression was detected by Western blotting. And the density of SLC7A5 immune-reactive bands was analyzed by using ACTB expression as a loading control (n=3). (*: $p < 0.05$)

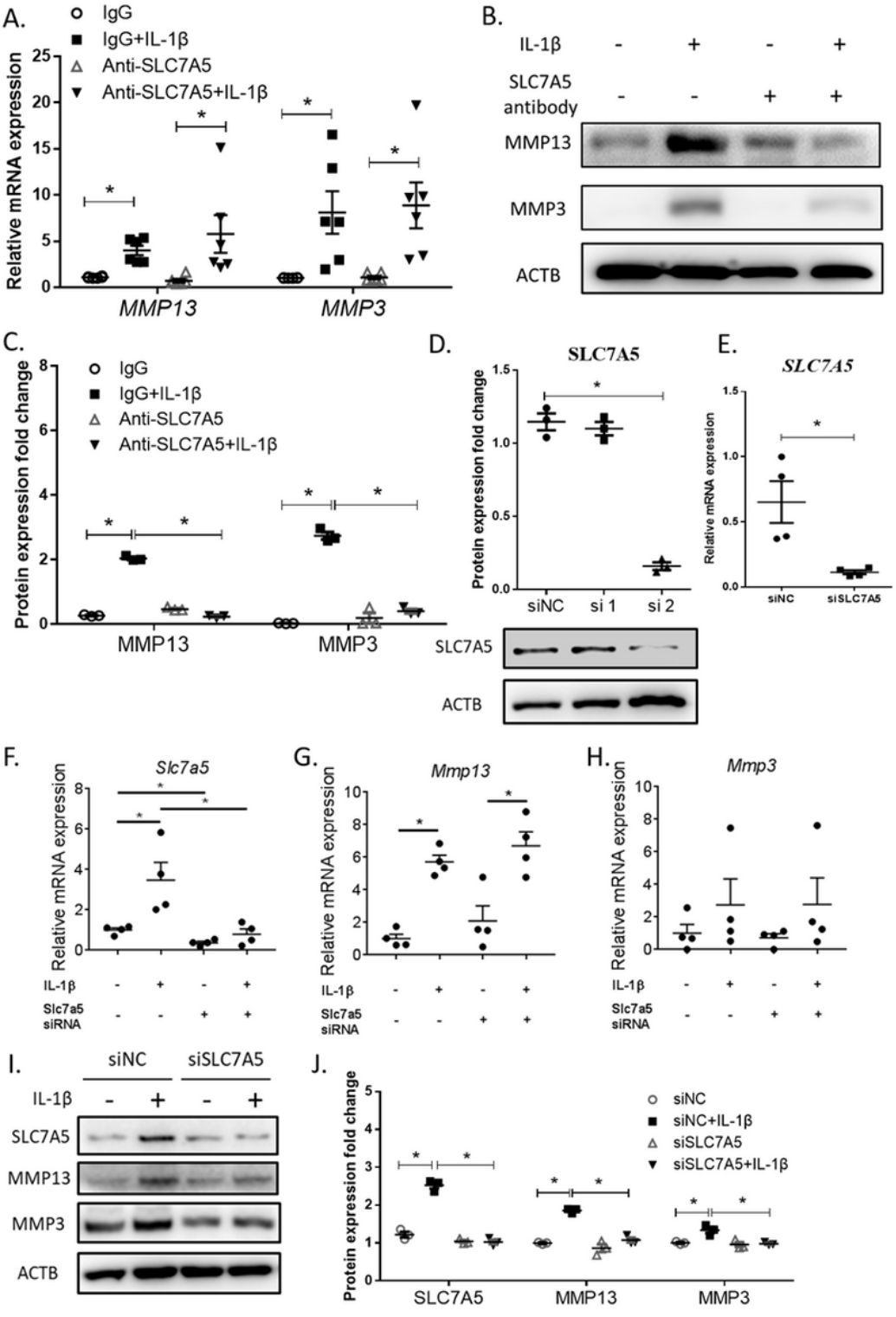

Figure 3

Blocking SLC7A5 function by mono-antibody or RNAi could affect only protein expression level of MMP3 and MMP13. A. The mRNA expression of MMP13 and MMP3 in FLS from RA patients. The cells were first treated with SLC7A5 monoclonal-antibody or isotype IgG for 4 hours and then treated with 20ng/mL IL-1β for 8 hours. The cells were collected for RT-qPCR assay, the expression of genes was detected by RT-qPCR (n=3). B. The representative protein expression of MMP13 and MMP3 in FLS from RA patients. The cells

were first treated with SLC7A5 monoclonal-antibody or isotype IgG for 4 hours and then treated with 20ng/mL IL-1β for 24 hours, and the protein was collected and detected by Western blotting. C. The density of MMP13 and MMP3 immune-reactive bands were analyzed by using ACTB expression as a loading control (n=3). D-E. The RNAi efficiency of SLC7A5 in FLS from RA patients. The cells were transfected with siRNA (NC, siSLC7A5-1 or 2) for 24 hours and then the protein and RNA were collected. The expression was detected by Western blotting (D) and RT-qPCR (E). F-H. The mRNA expression of SLC7A5, MMP13, and MMP3 in FLS from RA patients. The cells were first transfected with siRNA (NC, siSLC7A5) for 24 hours and then treated with 20ng/mL IL-1β for 8 hours. The cells were collected for RT-qPCR assay, the expression of genes was detected by RT-qPCR (n=4). I-J. The protein expression of SLC7A5, MMP13, and MMP3 in FLS from RA patients. The cells were first transfected with siRNA (NC, siSLC7A5) for 24 hours and then treated with 20ng/mL IL-1β for 24 hours. The density of MMP13 and MMP3 immune-reactive bands were analyzed by using ACTB expression as a loading control (n=3). (*, $p < 0.05$)

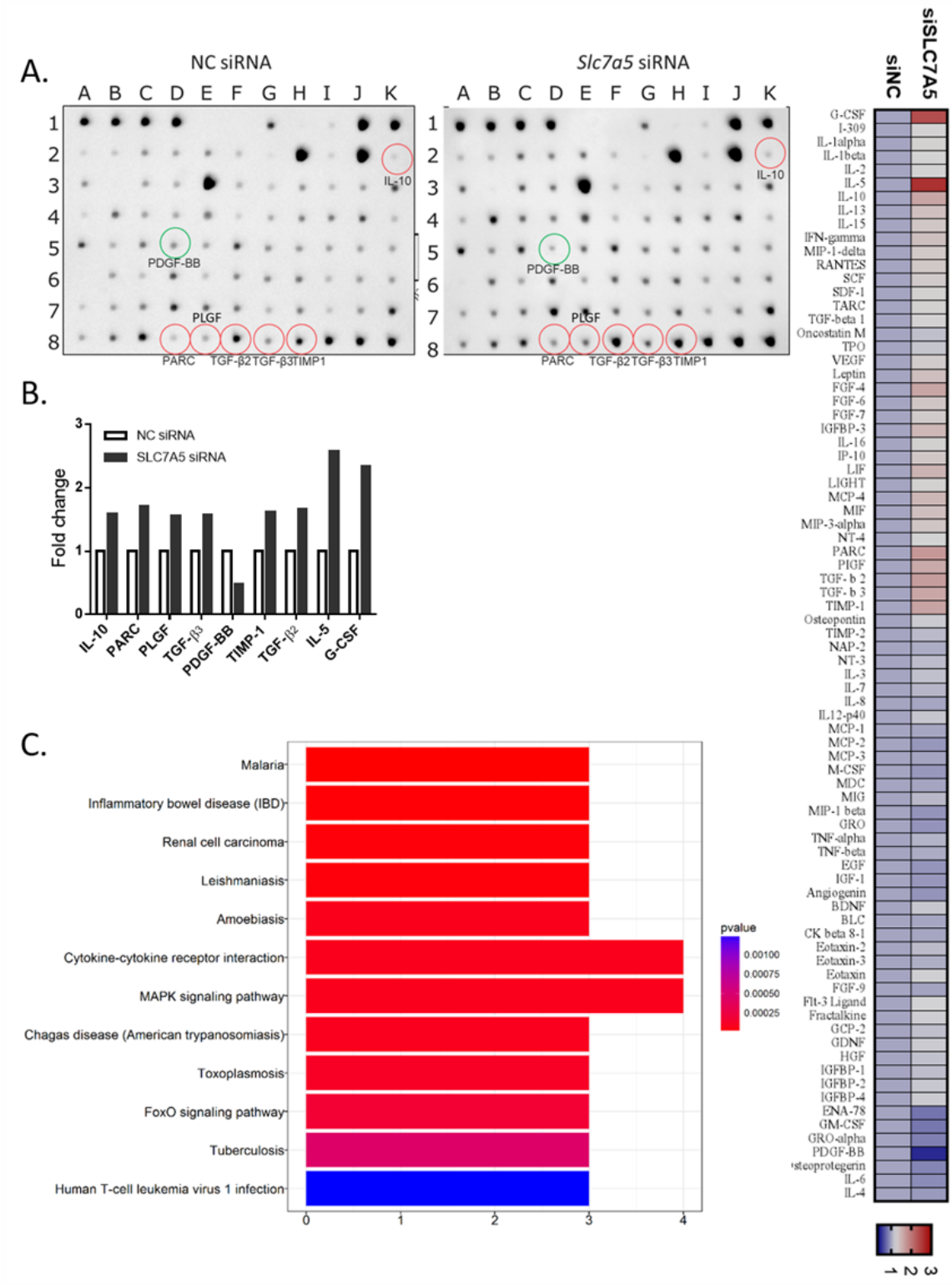

Figure 4

SLC7A5 would affect the production of cytokines and chemokines in RA FLS. A. Image of cytokine array result detecting protein production in supernatants from RA FLS transfected with siRNA for 48h. Two membranes were incubated with either negative control or SLC7A5 siRNA 48h transfected RA FLS supernatant. All the cytokines and chemokines fold change were shown in the heat map. B. Semi-quantitative data showing altered cytokine expression (fold change beyond ±1.5) in RA FLS supernatants

from after siRNA treatment for 48h. C. The differentially expressed cytokines and chemokines related pathways as analyzed by KEGG.

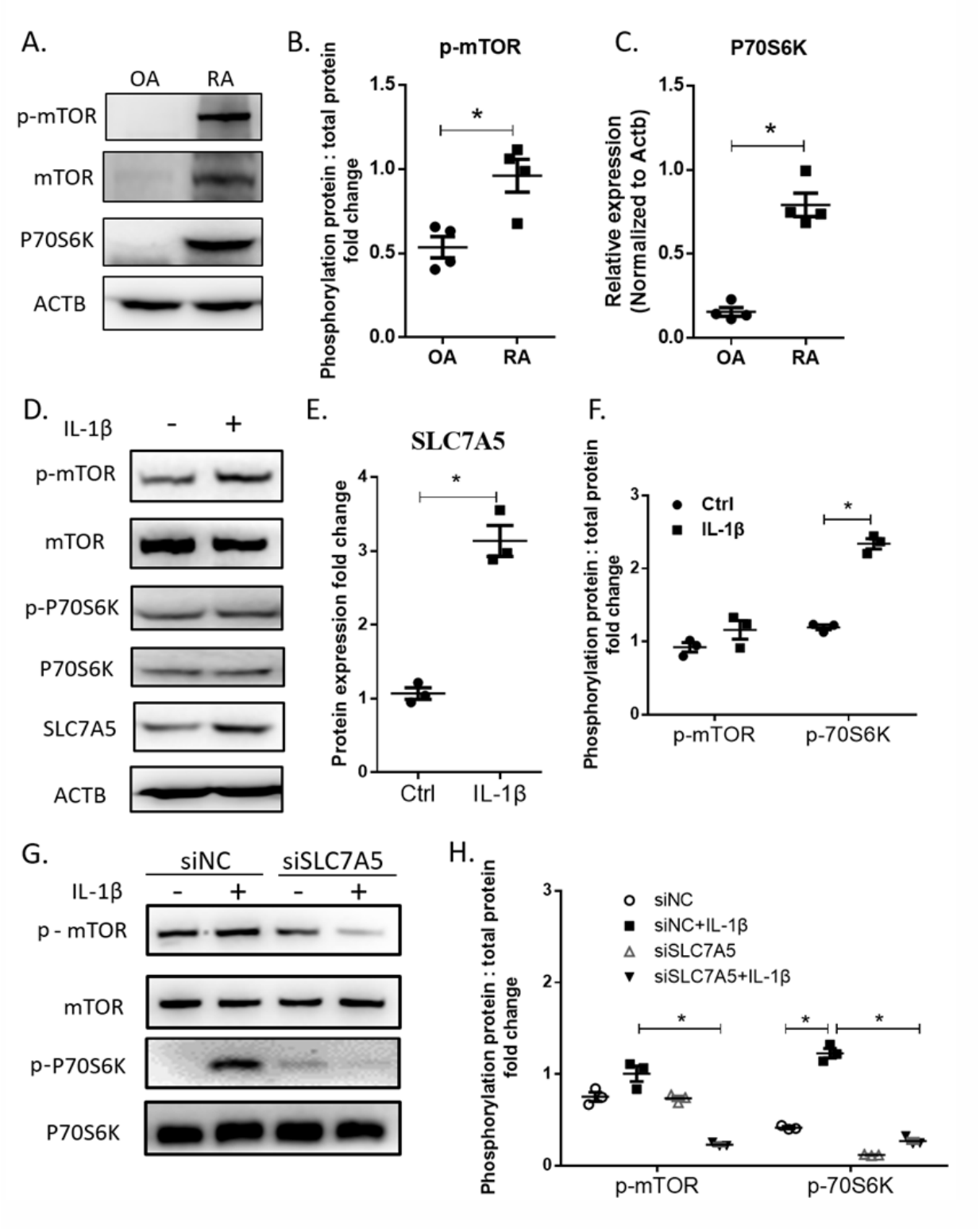

Figure 5

Up-regulated SLC7A5 activated mTOR-P70S6K signal pathway and enhanced MMP3 and MMP13 expression in FLS from RA patients. A-C. The protein expression of p-mTOR, mTOR, P70S6K and SLC7A5 in synovial tissue from OA and RA patients detected by Western blotting (n=4). D. The representative

protein synthesis pathway activation in FLS. The FLS were treated with 20ng/mL IL-1β for 4 hours, and then the protein was collected and detected by Western blotting. E. The density of SLC7A5 immune-reactive bands was analyzed by using ACTB expression as a loading control. F. The phosphorylation protein ratio fold change of mTOR and P70S6K were analyzed by using total protein expression of their own as a control (n=3). G. The protein synthesis pathway (mTOR-P70S6K) activation was inhibited by RNAi of SLC7A5 in FLS. The cells were treated with siNC or siSLC7A5 for 24 hours and then treated with 20ng/mL IL-1β for another 4 hours. The protein was collected and detected by Western blotting. H. phosphorylation protein ratio fold change of mTOR and P70S6K were analyzed by using total protein expression of their own as a control (n=3). (*: $p < 0.05$)

Supplementary Files

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