Online supplement to Angiotensin-converting Enzyme 2, a SARS-CoV-2 receptor, Is Upregulated by Interleukin 6 through STAT3 signaling in Synovial Tissues [letter], *The Journal of Rheumatology*, doi:10.3899/jrheum.200547

Supplementary Materials

- Methods
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Methods

Study design

This study was approved by the clinical ethics committees of Hiroshima University Hospital, Dohgo Spa Hospital and Ehime University Proteo-Science Center and Graduate School of Medicine and was conducted at these institutions (approval number: E-668; approval date: 01/02/2017). All experiments were performed in accordance with the approved guidelines. Synovial tissues were collected from 16 rheumatoid arthritis (RA) patients that fulfilled the classification criteria of the 1987 American College of Rheumatology (Arthritis and Rheum. 1988; 31: 315–324) and 3 osteoarthritis (OA) patients, who underwent total joint replacement, after obtaining informed and signed consent forms. Patients were not involved in the design, conduct, reporting, or dissemination of our research. Active synovitis was defined by the characteristics of multi-layered lining (hyperplasia), mesenchymoid transformation, palisading appearance of synovial lining and proliferation of blood vessels, all detected with hematoxylin and eosin (H & E) staining.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted and purified from synovial tissue (7 samples from 7 different RA patients) and cultured cells using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), followed by cDNA synthesis using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara-Bio, Kusatsu, JAPAN). RT-qPCR using Brilliant II SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA, USA) was outperformed with a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The upstream and downstream primer sequences for angiotensin-converting enzyme 2 (ACE2) were 5′- GAT TCT TTT TGG GGA GGA GGA -3′ and 5′- CTC CGG GAC ATC

CTG ATG -3′, respectively. The upstream and downstream primer sequences for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH, used as a control) were 5′- AAG GTC ATC CCA GAG CTG AA -3′ and 5′- CTG CTT CAC CAC CTT CTT GA -3′, respectively.

Immunohistochemistry

Sections obtained from formalin-fixed paraffin-embedded tissues (a total of 9 samples from 6 different RA patients and 3 different OA patients) were used for immunohistochemistry. Following deparaffinization and antigen retrieval in Tris-EDTA buffer (pH 9.0), the specimens were first incubated with primary antibodies (anti-ACE2, rabbit polyclonal, Bioss Antibodies Inc., Woburn, MA, USA), and then with the APconjugated secondary antibodies. The samples were then visualized using ImmPACT Vector Red Alkaline Phosphatase Substrate (Vector Laboratories, Burlingame, CA, USA), and the slides were counterstained with hematoxylin solution.

Purification and culture of fibroblast like synoviocytes (FLS)

To obtain FLS, synovial tissues samples from three different RA patients were minced and incubated with 1mg/ml collagenase/dispase (Sigma-Aldrich, Tokyo, JAPAN) in PBS (pH7.2) for 1 hour at 37 °C. The tissue samples were then filtered, washed, and cultured. During culture, the supernatant was replaced frequently to remove nonadherent cells. The FLS were cultured in DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, JAPAN) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and penicillin/streptomycin (FUJIFILM Wako Pure Chemical Corporation). Cultured cells at passages 3 through 6 were used for the experiments. Prior to analyses, cells were placed in DMEM with 0.5% FBS as serum starvation for >6 hours before the addition of recombinant human cytokines. Then, Interlukin-6 (IL-6) (100 ng/ml) and IL-6Rα (100 ng/ml) or TNFα (50 ng/ml) (BioLegend, San Diego, CA, USA) were added.

Western blot analysis

Cells were plated in six-well plates and washed with PBS before collection. Protein from cultured cells was processed using a SuperSep Ace 10% precast gel (FUJIFILM Wako Pure Chemical Corporation) and transferred to a PVDF membrane. Membranes were probed with anti-ACE2 (Bioss, rabbit polyclonal), anti-STAT3 Phospho Tyr705 (Biolegend, mouse monoclonal, clone. 13A3-1), anti-STAT3 (Bioss, rabbit polyclonal) and anti-β-actin (Sigma-Aldrich, mouse monoclonal, clone. AC-15) antibodies. HRP conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were then added. HRP activities were detected with ECL prime reagents (GE Healthcare, Tokyo, JAPAN) and imaged on an Image Quant LAS 500 (GE Healthcare).

Transfection experiments using siRNA

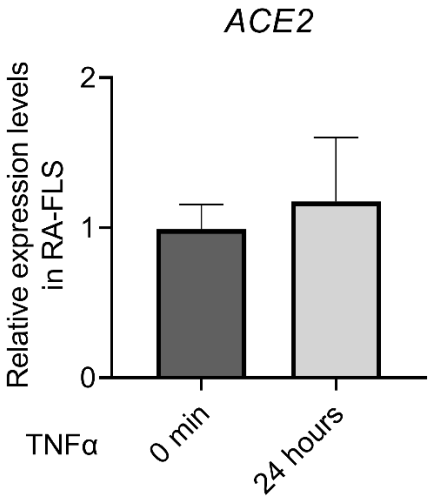
A total of 3 × 104 FLS per well in 12-well plates were transfected with siRNA at 20 pmol using ScreenFect siRNA (ScreenFect GmbH, Eggenstein-Leopoldshafen, Germany), according to the manufacturer's protocol. The siRNA targeting STAT3 (FlexiTube siRNA, SI02662898) and negative control (Allstar Negative Control siRNA) were purchased from QIAGEN (Shanghai, China).

Statistical analysis

All graphs show the results from one representative experiment though multiple repetitions were performed. The significance of the difference between paired groups was determined by Student's t-test. The differences among three or four groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test. Data processed and analyzed using GraphPad Prism 8 software (Graph Pad Software Inc., La Jolla, CA, USA).

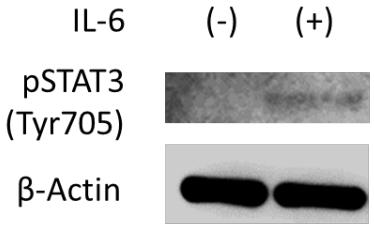
Supplementary figures

Figure S1. ACE2 expression was not induced by TNF stimulation.



RA-FLS were stimulated by recombinant human TNFα (50 ng/ml) for 24 hours. Cells were then harvested and RT-qPCR was performed (n=3). The results showed that TNF stimulation for 24 hours only slightly affected ACE2 expression levels and were not statistically significant.

Figure S2. Western blot analysis for detecting phosphorylation of STAT3 by IL-6 stimulation.



RA-FLS were stimulated by recombinant human IL-6 (100 ng/ml) combined with IL-6Rα (100 ng/ml) for 15 minutes. Then, cells were harvested, and western blot analysis was performed. The result showed that IL-6 signaling led to phosphorylation of STAT3.