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Anti-arthritis Effect of Anti-chitinase-3-like 1 Antibody Through Inhibition of MMP3

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ABSTRACT

Chitinase-3-like 1 (CHI3L1) is a key factor in regulating inflammatory processes and development of rheumatoid arthritis (RA) since is highly produced by synoviocytes and macrophages in the development RA. Collagen-induced arthritis (CIA) model is the most widely used because its pathogenesis is similar to human RA. Thus, we aimed to investigate if anti-CHI3L1 antibody could reduce RA development in the CIA model. To induce CIA, DBA1/J mice were immunized with a type II bovine collagen emulsion in complete Freund's adjuvant, and boosted type II bovine collagen. THP-1 and MH7A cells were used for pro-inflammation responses. Anti-CHI3L1 Ab treatment reduced the RA clinical score and paw thickness of mice. Inflammation-induced matrix metalloproteinase 3 (MMP3) expression was reduced by inhibiting CHI3L1, and MMP3 knockdown suppressed the expression of RA-related inflammatory cytokines in LPS-treated THP-1 and MH7A cells. Our findings suggest that anti-CHI3L1 Ab showed significant anti-arthritic effects by inhibiting MMP3 expression.

Keywords: Chitinase-3-like protein 1; Antibodies; Experimental arthritis; Rheumatoid arthritis; Matrix metalloproteinase 3; Caspase 1

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, destructive inflammatory autoimmune disease characterized by bone erosion, joint deformities, and tissue injury (1). Initiated by a combination of environmental and genetic factors, RA pathogenesis involves the activation of various immune cells, including macrophages and synoviocytes (2). This leads to a cascade of inflammatory responses within the joints, culminating in the destruction of cartilage and bone. Inflammation is a central driver of RA, and numerous disease-modifying anti-rheumatic drugs have been developed to effectively manage the disease by targeting and reducing these inflammatory responses.

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

AD, Alzheimer's disease; CCP, cyclic citrullinated peptide; CHI3L1, chitinase-3-like 1; CIA, collagen-induced arthritis; COX-2, cyclooxygenease-2; CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; ESR, erythrocyte sedimentation rate; FDA, Food and Drug Administration; iNOS, inducible nitric oxide synthase; Ly6g, lymphocyte antigen 6 family member G; mAb, monoclonal antibody; MMP, matrix metalloproteinase; MRC1, mannose receptor C-type 1; PGE2, prostaglandin E2; RA, rheumatoid arthritis; RF, rheumatoid factor; ROC, receiver operating characteristic; RT-qPCR, quantitative real-time polymerase chain reaction.

Author Contributions

Conceptualization: Kim DH, Yu JE; Data curation: Lee DH, Kim MJ, Jeon SH; Formal analysis: Lee DH, Kim MJ, Jeon SH; Funding acquisition: Hong JT; Investigation: Yun J, Son DJ; Methodology: Yun J, Son DJ, Kim TH; Project administration: Lee YS; Resources: Kim B, Yong YJ, Lim YS, Khalid AM; Software: Kim B; Supervision: Han SB, Lee YS, Hong JT; Validation: Kim DH; Visualization: Han SB; Writing - original draft: Kim DH, Yu JE; Writing - review & editing: Kim DH, Yu JE, Khalid AM, Lee YS, Hong JT.

Chitinase-3-like 1 (CHI3L1), an 18 glycosyl hydrolase gene family, is expressed and secreted in various cell types, including macrophages, neutrophils, epithelial cells, smooth muscle cells, and chondrocytes (3,4). CHI3L1 has been associated with many diseases such as osteoarthritis, liver fibrosis, inflammatory bowel disease, bacterial septicemia, atherosclerotic cardiovascular disease, and various neurological diseases, including Alzheimer's disease (AD), amyotrophic lateral sclerosis, multiple sclerosis, and schizophrenia (4-6). A recent meta-analysis indicated that the serum/plasma level of CHI3L1 was significantly higher in patients with RA than in controls and was correlated with RA activity, including the 28-joint Disease Activity Score (DAS28), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) levels, and rheumatoid factors (RFs) (7). Our previous studies revealed that inhibiting CHI3L1 reduced the levels of various inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in multiple inflammation-related disease models such as phthalic anhydride-induced atopic disease and LPS-induced acute liver injury models (8-11). These results suggested that CHI3L1 could be critical for RA development.

Matrix metalloproteinases (MMPs) are involved in the pathogenesis of RA through extracellular matrix degradation in the cartilage (12). MMP3, a member of the stromelysin family, possesses broad substrate specificity, cleaving various collagens, glycoproteins, and other extracellular matrix molecules (13). MMP3 is expressed by key cell types involved in RA, including chondrocytes, fibroblast-like synoviocytes, and macrophages. Previous studies have demonstrated the involvement of MMP3 in cartilage destruction (14-18). Furthermore, several studies have reported significant elevations in serum MMP3 levels in patients with RA, particularly in those with moderate to severe disease activity. For instance, a study of 151 Chinese RA patients demonstrated significantly higher serum MMP3 levels in patients with moderate and severe disease, as classified by the 28-joint DAS28, compared to healthy controls (19). Another study by Hattori et al. (20) also observed a positive correlation between serum MMP3 levels and DAS28 score in RA patients, with the highest levels observed in the high-DAS28 group. The interplay between CHI3L1 and MMPs has also been documented in inflammatory conditions. Recombinant CHI3L1 protein dose-dependently stimulated the production of MMP9 by LPS-treated mouse pulmonary macrophages (21). Additionally, a correlation between CHI3L1 levels and the levels of MMP1 and MMP3 has been observed in synovial fluid and cartilage culture supernatants from patients with osteoarthritis (22).

In our previous study, we produced and selected CHI3L1-targeting monoclonal Abs (mAbs) (23). We used phage display to develop novel mAbs targeting human CHI3L1. Human synthetic Ab phage display libraries were panned against recombinant CHI3L1 protein, leading to the identification of 7 unique Fabs (H1, H2, H3, H4, H5, H6, H7). Among these, we selected the high-affinity human H1 clone mAb from the synthetic Fab phage display libraries. The H1 clone has favorable biophysical properties, including high affinity, thermal stability, and resistance to aggregation. Additionally, H1 showed remarkable success in preventing the growth and metastasis of cancer cells *in vitro*, Thus, we selected H1 clone for studies in this experiment. Based on our previous findings demonstrating that an optimal anti-CHI3L1 Ab candidate effectively inhibited lung tumor growth and metastasis through anti-inflammatory mechanisms (24), we investigated the potential therapeutic effects and underlying mechanisms of this anti-CHI3L1 Ab in the context of RA.

MATERIALS AND METHODS

Materials

The anti-CHI3L1 Abs obtained from the H1 clone was produced as previously described (23).

Animals

Six-week-old male DBA/J1 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were maintained in accordance with the guidelines of the Ministry of Food and Drug Safety of Korea and the regulations for the care and use of laboratory animals of the Animal Ethics Committee of Chungbuk National University (CBNUA-1425-20-01). The mice (n=5/cage) were maintained in a room with a constant temperature of 22°C±1°C, relative humidity of 55%±10%, and a 12-h light/dark cycle, and were fed standard rodent chow (Samyang, Seoul, Korea) and purified tap water.

Collagen-induced arthritis (CIA) model

CIA model was performed as described previously (25). An emulsion was formed by dissolving 2 mg/ml bovine collagen type II (Chondrex, Inc., Redmond, WA, USA) and 2 mg/ml complete Freund's adjuvant (Chondrex, Inc.) (vol:vol=1:1). For CIA induction, 8-wk-old male DBA1/J mice were immunized by intradermal injection with 100 μl of the emulsion at the base of tail. Twenty days after the first immunization, mice were received an intraperitoneal booster injection with 100 μl of collagen type II (defined as day 0). For anti-CHI3L1 Ab therapy experiment, CIA-induced mice received intraperitoneal injections of vehicle (0.9% sodium chloride injection solution; JW Pharmaceuticals, Gwacheon, Korea), anti-CHI3L1 Abs (2.5 and 5 mg/kg), or anti-TNF-α Abs (5 mg/kg) 3 times per week at a volume of 100 μl from day 0 to day 24. The severity of arthritis was assessed using a semi-quantitative scoring system (0–4) as follows: 0, normal; 1, redness and/or swelling in one joint; 2, redness and/or swelling in more than one joint; 3, redness and/or swelling of the entire paw; and 4, deformity and/or ankylosis. After adding the scores from the 4 paws, the highest total possible score for each mouse was 16 by the methods described by Ban et al. (25).

Radiological assessment

Before sacrificing the mice, the joints of their hind limbs were imaged using an X-ray apparatus (REX-525R; Listem, Wonju, Korea) with an industrial X-ray film (Kodak Photo Film, Rochester, NY, USA) to assess joint damage. The X-ray apparatus was operated at 220 V with a 40 V peak, 0.05 s exposure time, and 100 cm tube-to-film distance for anterior-posterior projection.

Immunohistochemical assay

Immunohistochemistry was performed on paraffin sections of paw joint tissues obtained from each mouse on day 9 (5 mice/each experimental group). For histological processing, paws were fixed in a phosphate buffer containing 10% formaldehyde and decalcified with EDTA. Paws were processed into paraffin blocks using routine methods. The center of each ankle joint was sliced into 5-μm-thick sagittal slices and stained with H&E, safranin O, anti-cyclooxygenease-2 (COX-2), anti-inducible nitric oxide synthase (iNOS), F4/80, lymphocyte antigen 6 family member G (Ly6g) and mannose receptor C-type 1 (MRC1) Abs. Paraffin-embedded mouse tissue sections were blocked for 1 h with 2% normal serum diluted in PBS. Subsequently, sections were incubated with specific primary Abs overnight at 4°C in blocking serum. After 3 washes with PBS for 5 min, the slides were incubated in biotinylated secondary Ab for 1 h. The slides were washed in PBS, followed by formation of the avidin-

biotin-peroxidase complex (Vector Laboratories, Inc., Burlingame, CA, USA). After washing the slices, the peroxidase reaction was developed using diaminobenzidine and peroxide, and the sections were counterstained with hematoxylin. Histological images were taken under a microscope at ×200 magnification (VS200; Olympus, Tokyo, Japan). Detailed information is presented in **Supplementary Table 1**.

Western blot analysis

Western blot analysis was performed as previously described (26). Specially, joint tissue was processed by pulverization in liquid nitrogen using a mortar and pestle, followed by aliquoting into 1.5 ml tubes, homogenization in lysis buffer, and centrifugation to collect the supernatant. The membrane was incubated with the following specific primary Abs against iNOS, COX-2, MMP3, MMP9, MMP13, procaspase-1, CD86, MRC1 and β-actin. The relative density of the protein bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). β-actin was used as a loading control. Detailed Ab information is presented in **Supplementary Table 1**.

Quantitative real-time PCR (RT-qPCR)

RT-qPCR was performed as previously described (26). Briefly, total RNA was extracted from the cell and tissue samples using a Ribo^{EX} RNA Extraction Kit (GeneAll Biotechnology, Seoul, Korea), and cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using specific primers with the StepOnePlus™ PCR System (Applied Biosystems). The expression levels obtained for the target gene were normalized to 18S and quantified relative to the expression in control samples. Primer sequences were obtained from previous studies (9,27-34). Detailed primer sequence information is presented in **Supplementary Table 2**.

ELISA

The levels of cytokines, nitric oxide, and prostaglandin E_2 (PGE_2) as well as CHI3L1 were evaluated using ELISA kits according to the manufacturer's protocol. Detailed ELISA kit information is presented in **Supplementary Table 1**.

Cell culture and transfection

THP-1 human monocytic cells were obtained from the American Type Culture Collection (Manassas, VA, USA). MH7A, a human rheumatoid fibroblast-like synoviocyte line obtained from the Riken Cell Bank (Tsukuba, Japan), was kindly provided by Dr. Eun-Yi Moon (College of Department of Bioscience and Biotechnology, Sejong University, Seoul, Korea) and Dr. Yong-Yeon Cho (College of Pharmacy, Catholic University, Seoul, Korea). Cells were grown in RPMI 1640 medium with l-glutamine and 25 mM HEPES buffer (Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were transfected with MMP3 siRNA or its controls (Origene, Rockville, MD, USA) using Lipofectamine RNAiMAX reagent in Opti-MEM according to the manufacturer's instructions (Invitrogen). THP-1 cells were differentiated into macrophages after incubation with 100 ng/ml PMA for 24 h. They were then incubated with rhCHI3L1 (250 and 500 ng/ml, R&D Systems, #2599-CH) or anti-CHI3L1 Ab (250 and 500 ng/ml) for 24 h.

Human samples

Human serum samples from patients with RA and healthy controls (25 samples each) were provided by the Biobank of Chungbuk National University Hospital and Gyeongsang National

University Hospital, which are members of the Korea Biobank Network. The patient group (n=25) had a mean age of 65.4 years (9/25 male and 16/25 female) and confirmed by visual examination (100% positive), ESR test (100% positive), CRP test (100% positive), RF test (100% positive) and anti-cyclic citrullinated peptide (CCP) Ab test (56% positive). The healthy control group consisted of 25 individuals with a mean age of 26.4 years (100% male). For the healthy control group, no RA-related test data were provided. All studies using human samples were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Chungbuk National University Medical Center (IRB No. CBNU-202003-BR-0019-01).

Microarray data analysis

The potential CHI3L1-regulated target genes were screened using 2 public microarray datasets (GSE13071 and GSE48780). The GSE13071 dataset containing data from the knee joint synovium of CIA mice was analyzed using GEO2R (http://www.ncbi.nlm.nih.gov/geo/info/geo2r.html) (35). Severe joint inflammation samples (n=3) and naïve samples (n=3) were selected to compare the differences in gene expression profiles in GSE13071 dataset (severe joint inflammation vs. naïve). Among the genes with differences, those with adjusted p-value <0.05 were used. The GSE48780 dataset containing data from synovial samples of human patients with RA with different disease activities was analyzed using GEO2R (36). Inflammatory (n=3) and non-inflammatory (n=8) knee synovial tissue samples were selected to compare the differences in gene expression profiles in GSE48780 dataset (inflammatory vs non-inflammatory). All genes with differences had an adjusted p-value of 1 and were therefore used regardless of significance. Genes that commonly upregulated or downregulated more than 2-fold between human patients with RA and mouse CIA models (if the gene ID was different but the gene symbol was the same, it was counted as one) were classified as the potential CHI3L1-regulated target genes.

Gene network analysis

The gene network between CHI3L1 and the potential CHI3L1-regulated target genes were analyzed by using the web-based gene-gene interaction analysis tool GeneMANIA (http://www.genemania.org) based on the large set of functional association data (gene-gene interactions based on attributions: co-expression, co-localization, genetic interactions, pathway, physical interactions, predicted interactions and shared protein domains). The predicted gene network is generated by gene-ontology base weighting methods (37).

Statistical analysis

The experiments were conducted in triplicate, and all experiments were repeated at least 3 times, with similar results. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). Group differences were analyzed using one-way ANOVA, followed by Tukey's multiple comparison test. All values are presented as the means ± SDs. Statistical significance was set at $p<0.05$.

RESULTS

Clinical correlation between CHI3L1 and human RA

Previous studies have confirmed the clinical association between CHI3L1 and RA through meta-analyses (7), and CHI3L1 upregulate the expression of inflammatory cytokines in the synovial fluid and serum of RA patients which could be significant associated with US Food

and Drug Administration (FDA) recommended biomarkers. Thus, we first analyzed the levels of CHI3L1 and other FDA-recommended biomarkers, such as Abs against CCP, CRP, and RFs, in the serum of patients with RA using ELISA. The serum levels of CHI3L1, anti-CCP Ab, CRP, and RF were significantly higher in patients with RA than in healthy controls (**Fig. 1A**). We further analyzed the receiver operating characteristic (ROC) curve for diagnostic reference. The RA diagnostic cutoff value, sensitivity, specificity, and area under the curve of CHI3L1 were 18.63, 92%, 92%, and 0.9552, respectively (**Fig. 1A**). Those for anti-CCP Ab were 1,097, 96%, 96%, and 0.9952, respectively (**Fig. 1A**); for CRP: 15.02, 96%, 96%, and 0.9888, respectively; and for RF: 48.82, 76%, 76%, and 0.8656, respectively (**Fig. 1A**). Thus, the ROC analysis indicated that CHI3L1 is a good diagnostic reference for RA. Spearman's correlation test was used to evaluate the correlation among CHI3L1, anti-CCP Ab, CRP, and RF in patients with RA. Serum CHI3L1 levels were positively correlated with serum RA (r=0.4512, p=0.0236; **Fig. 1B**). However, serum CHI3L1 levels were not correlated with serum anti-CCP Ab (r=0.07674, p=0.7154) or CRP levels (r=0.2291, p=0.2706) (**Fig. 1B**). These findings suggested that CHI3L1 may be a better biomarker associated with the RA compared to other FDA recommended biomarkers.

CHI3L1 is associated with inflammation *in vitro*

Our previous studies have shown that CHI3L1 is involved in inflammatory responses, including atopic dermatitis, neurological diseases, and the cancer microenvironment

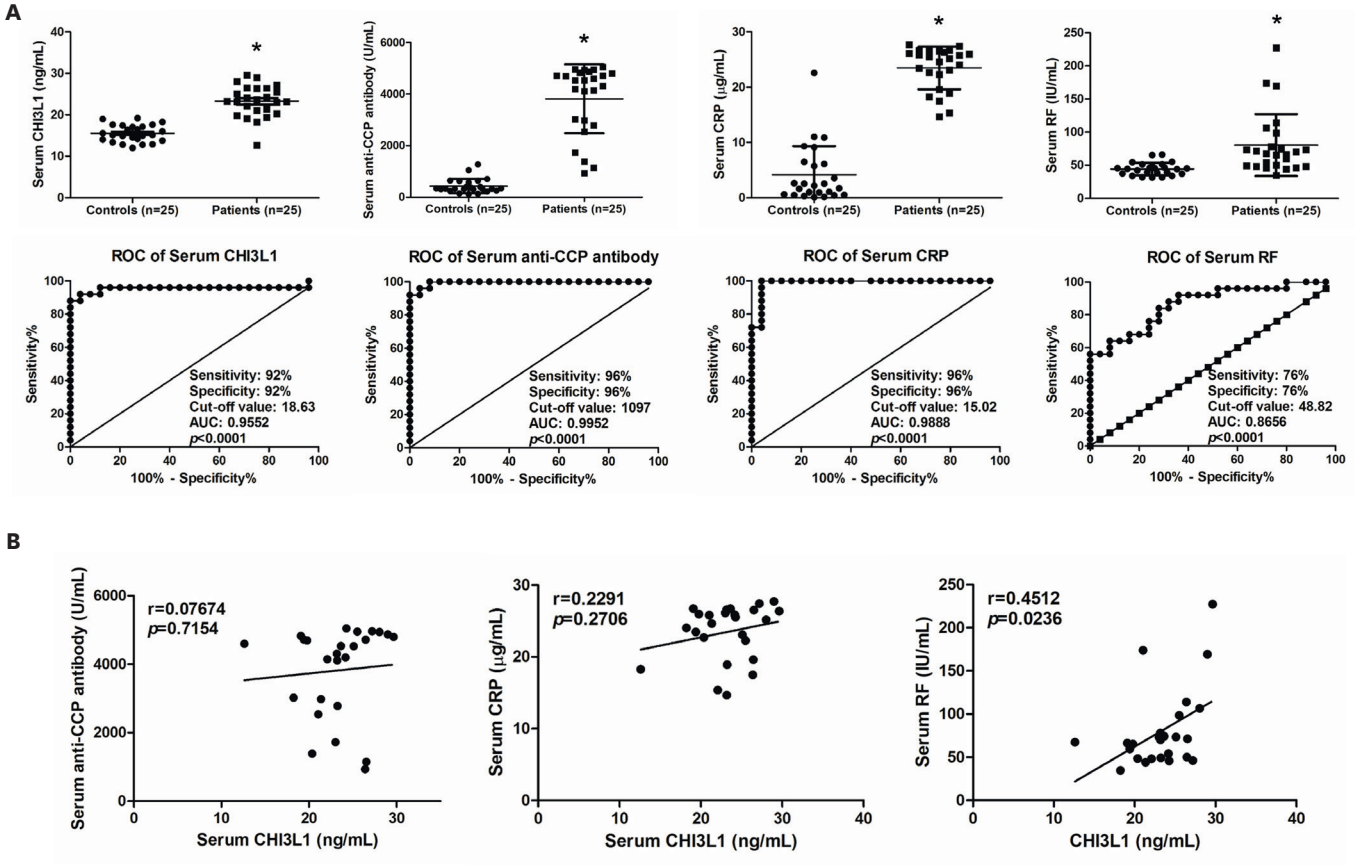

Figure 1. Serum analysis of CHI3L1, anti-CCP Ab, CRP and RF in patients with RA. (A) The serum levels and ROC curve of CHI3L1, anti-CCP Ab, CRP and RF in patients with RA and healthy controls (n=25 each). (B) Spearman correlation test results between anti-CCP Ab, CRP and RF for CHI3L1.
Healthy control vs. patients with RA: *p<0.05.

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(8-10,23,27). So far, our results suggest that CHI3L1 expression is also correlated with RA. Hence, to determine whether CHI3L1 alone can induce inflammatory responses, we evaluated the secretion of RA-related cytokines by THP-1 and MH7A cells. Recombinant CHI3L1 treatment increased the levels of TNF-α, IL-1β, IL-18, and CCL2 in THP-1 and MH7A cells (**Fig. 2A**). We also analyzed these cytokines when CHI3L1 was blocked with an anti-CHI3L1 Ab. The levels of the 4 RA-related cytokines were decreased in anti-CHI3L1 Ab-treated THP-1 and MH7A cells (**Fig. 2B**). These results indicate that CHI3L1 may contribute to inflammatory responses in RA pathogenesis.

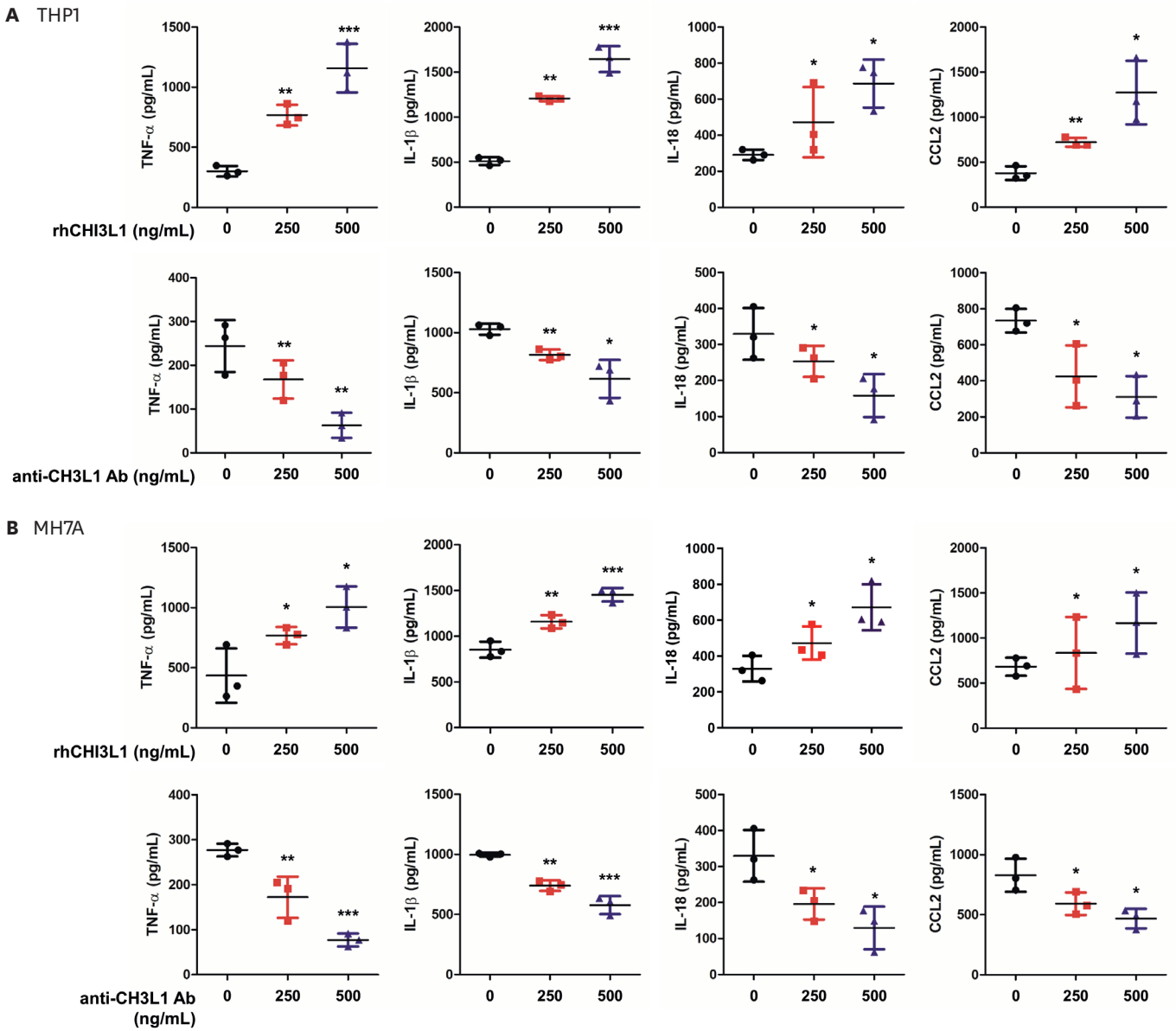

Figure 2. CHI3L1 regulates RA-related cytokine release. (A, B) PMA-treated THP-1 and MH7A cells were cultured for 24 h in presence of rhCHI3L1 or anti-CHI3L1 Ab. The levels of RA-related cytokines, TNF-α, IL-1β, IL-18 and CCL2, were assessed using ELISA.
Non-treated vs. treated with rhCHI3L1 or anti-CHI3L1 Ab: *p<0.05; **p<0.01; ***p<0.001.

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Anti-CHI3L1 Ab therapy reduces RA pathogenesis in a CIA mouse model

To figure out the inhibitory effect of anti-CHI3L1 Abs, we analyzed the effect of anti-CHI3L1 Abs on RA pathogenesis by blocking CHI3L1 in a CIA mouse model. In addition, we administered anti-TNF-α Abs, which is widely used as an RA treatment target, and attempted to compare its effectiveness with CHI3L1 Abs. As shown in **Fig. 3A**, the severity of arthritis was increased over time until the end of the experiment. Mice treated thrice weekly with anti-CHI3L1 or anti-TNF-α Abs for 3.5 wk showed a sustained reduction in the semi-quantitative RA assessment score (clinical score), which was significant from days 3 to 24. Disease severity, including paw thickness, was significantly reduced upon anti-CHI3L1 Ab treatment in the CIA mice (**Fig. 3A-C**). When comparing to the anti-TNF-α Abs, treatment with anti-CHI3L1 Abs showed similar inhibitory effects. Cartilage pathology was assessed via joint histological analysis of ankle sections stained with H&E and safranin O. Safranin O is a cationic dye and used to stain matrix proteoglycans to study cartilaginous tissue structural integrity. Cartilage is also generally considered the main target tissue in RA. Cartilage becomes damaged as a result of inflammation and hyperplastic synovial membrane damage caused by systemic autoimmune events in the joint. Thus, we examined tissue damages with safranin O staining. Histopathological examination of the ankle joints of CIA mice showed cartilage destruction with chondrocyte death, as well as the presence of synovial infiltrates and exudates. Treatment with both doses of the anti-CHI3L1 Ab led to a reduction in chondrocyte death, cartilage erosion, and synovial exudates (**Fig. 3B**). These data showed that reducing effects of anti-CHI3L1 Ab on the cartilage destruction with chondrocyte death, as well as the presence of synovial infiltrates and exudates could resulted in the mitigation of arthritis symptoms of anti-CHI3L1 Ab. Furthermore, CHI3L1 protein levels were significantly decreased in the ankle joint tissues and sera of CIA mice after the administration of anti-

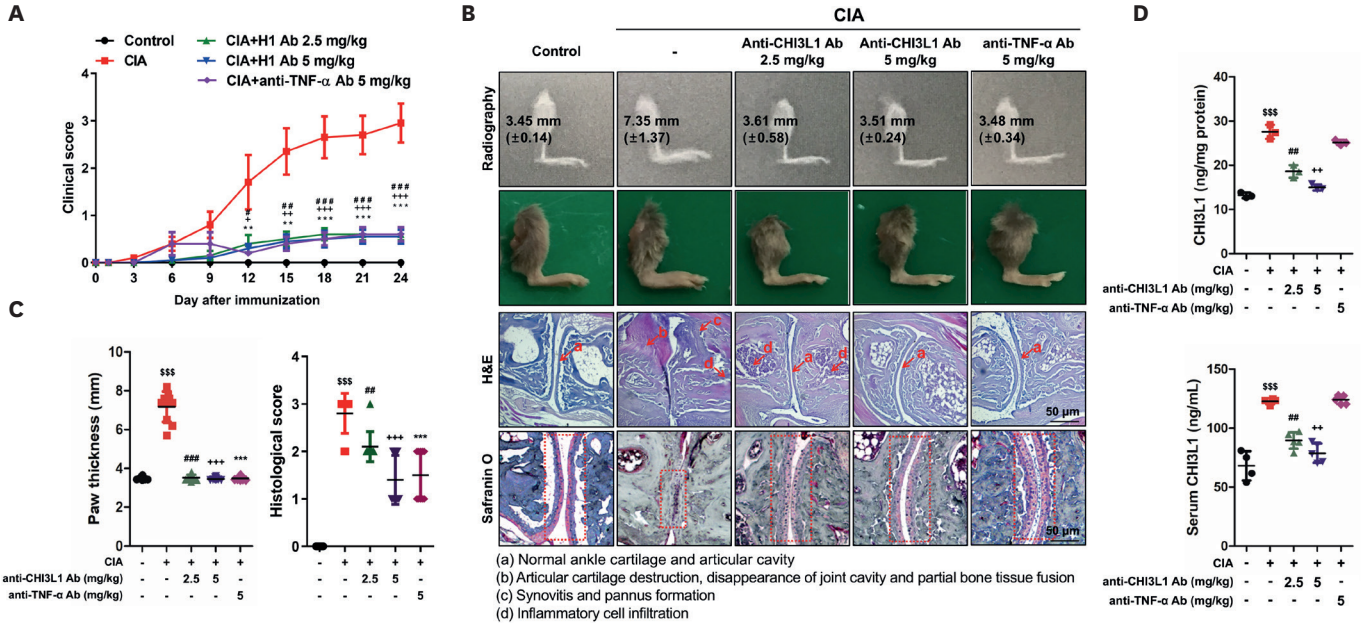

Figure 3. Anti-CHI3L1 Ab therapy reduced RA development. (A) Clinical score of arthritis in CIA mice treated with anti-CHI3L1 Ab or anti-TNF-α Ab. (B) Morphological, radiographical and histological changes in CIA mice after 24-day treatment. (C) Bar graphs indicate the paw thickness and clinical score. The paw thickness of each mouse was measured with a vernier caliper (n=5). (D) Ankle joint tissues and sera of CIA mice were collected by sacrificing the mice a day after the 3-wk administration of anti-CHI3L1 Ab or anti-TNF-α Ab. The concentration of CHI3L1 in mouse ankle (upper panel) and serum (lower panel) (n=5).
CIA control vs. CIA treated with anti-CHI3L1 Ab 2.5 mg: $^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$.
CIA control vs. CIA treated with anti-CHI3L1 Ab 5 mg/kg: $^{+}p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$.
CIA control vs. CIA treated with anti-TNF-α Ab 5 mg/kg: $^{**}p<0.01$; $^{***}p<0.001$.
Non-CIA control vs. CIA control: $^{\$\$\$}p<0.001$.

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CHI3L1 Abs (**Fig. 3D**). The inhibitory effects on CHI1L1 levels were not found in the anti-TNF-α Ab treatment group, and histopathological effects of anti-CHI3L1 Ab were more effective compared to those by anti-TNF-α Ab treated group. The infiltration of immune cells into joints is a critical feature of RA pathogenesis. Therefore, we investigated the infiltration of immune cells into mouse ankle joint tissue samples. We found a decreased infiltration of F4/80+ macrophages and Ly6G+ neutrophils in CIA mice treated with anti-CHI3L1 Ab and anti-TNF-α Ab compared with in control CIA mice (**Supplementary Fig. 1A**). Cutolo et al. (38) revealed that an M1 (pro-inflammatory)/M2 (anti-inflammatory) macrophage imbalance may contribute to RA development and that an increase in the proportion of M2 macrophages could have a therapeutic effect on RA. We further analyzed the infiltration of M1 macrophages (iNOS+) and M2 macrophages (MRC1+) as well as the expression levels of M1 macrophage markers (iNOS and CD86) and M2 macrophage markers (MRC1 and ARG1) in the CIA model. The results indicated that the infiltration of M1 and M2 macrophages to the joints was decreased. Similarly, the expressions level of M1 and M2 macrophage markers were also reduced in CIA mice treated with anti-CHI3L1 or anti-TNF-α Abs compared to control CIA mice. However, the expression of M2 macrophage markers were more critically reduced (**Supplementary Fig. 1B-D**).

Anti-inflammatory effects of anti-CHI3L1 Ab on CIA mouse model

Immunohistochemical analysis revealed increased expression of the inflammatory markers iNOS and COX-2 in ankle joint tissue samples obtained from CIA mice, which were localized primarily in fibrotic regions surrounding the joints. In contrast, few areas were stained positive for COX-2 and iNOS in CIA mice treated with anti-CHI3L1 or anti-TNF-α Abs treated group (**Fig. 4A**). These inhibitory effects were accompanied by inhibition of iNOS and COX-2 expression (**Fig. 4B**). We also confirmed that LPS-induced iNOS and COX-2 expression was decreased in anti-CHI3L1 Ab-treated THP-1 and MH7A cells, and the inhibitory effects of these inflammatory responses were similar by anti-CHI3L1 and anti-TNF-α Abs (**Fig. 4C**). In addition, we investigated whether anti-CHI3L1 Abs could block the release of inflammatory mediators. For this purpose, we measured the levels of RA-related cytokines (TNF-α, IL-1β, IL-18, and CCL2), nitric oxide, and PGE_2 levels in the ankle joint tissue samples of the CIA model. We found a significant reduction in the mRNA levels of the RA-related cytokines and the levels of nitric oxide and PGE_2 after treatment with both doses of anti-CHI3L1 Abs in anti-CHI3L1 Ab-treated THP-1 and MH7A cells, and the inhibitory effects of these inflammatory responses were similar by anti-CHI3L1 and anti-TNF-α Abs (**Fig. 4D and E**).

MMP3 is associated with CHI3L1-mediated RA development

To identify the factors associated with anti-CHI3L1 Ab treatment in the CIA model, we first used publicly available data to identify potential CHI3L1-regulated target genes that were commonly upregulated or downregulated in patients with RA (GSE48780 dataset) and mouse CIA models (GSE13701 dataset) using GEO2R (**Fig. 5A**). The results showed that 24 genes were upregulated and 13 genes were downregulated in both human RA patients and mouse CIA model samples (**Supplementary Table 3**). We then analyzed whether these genes were associated with CHI3L1 using GeneMANIA. We found that 6 genes (upregulated: IL7R, NPL, MMP3, LBP, COL3A1, and CXCL9; downregulated: none) were correlated with CHI3L1 (**Supplementary Fig. 2**). Among these genes, MMP3 was selected based on a literature search. Previous studies have indicated that MMP3 is associated with inflammation in various diseases, including RA (13,39). Interestingly, a recent study reported that CHI3L1 is significantly correlated with MMP3 levels in the serum of patients with osteoarthritis (22). We also selected caspase-1, which is also related to CHI3L1 because recombinant CHI3L1

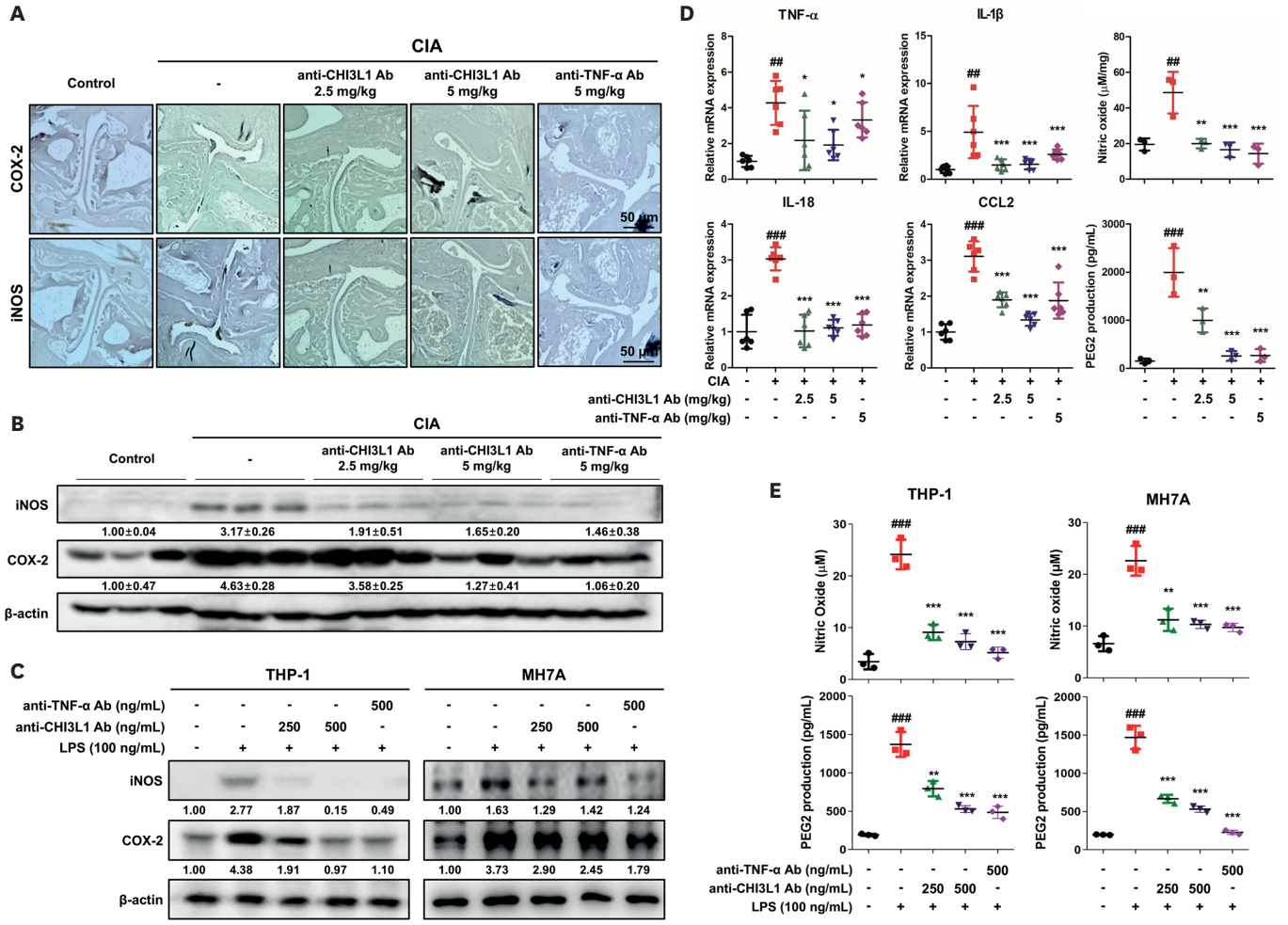

Figure 4. Anti-CHI3L1 Ab suppresses inflammatory responses in mouse CIA model. (A, B) Expression of iNOS and COX-2 in CIA ankle tissue samples by Western blot analysis and immunohistochemistry analysis. (C) PMA-treated THP-1 and MH7A cells were pretreated with anti-CHI3L1 Ab or anti-TNF-α Ab for 1 h. Then, cells were stimulated with LPS (100 ng/ml) for another 24 h. Expression of iNOS and COX-2 were analyzed by Western blot analysis. (D) mRNA Expression of TNF-α, IL-1β, IL-18 and CCL2 in mouse CIA ankle tissue samples by qPCR. Levels of nitric oxide and PEG2 in CIA ankle tissue samples by ELISA. (E) Levels of nitric oxide and PEG2 in *in vitro* samples by ELISA.
Non-CIA control vs. CIA control/non-treated control vs. LPS: $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$.
CIA control vs. CIA treated with anti-CHI3L1 Ab or anti-TNF-α Ab/LPS vs. LPS treated with anti-CHI3L1 Ab or anti-TNF-α Ab: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

treatment induced IL-1β and IL-18 levels *in vitro*. A previous study showed that caspase-1-deficient mice showed reduced synovial inflammation, cartilage damage, and bone erosion in a streptococcal cell wall fragment-induced chronic arthritis model (40). A clinical study revealed that active caspase-1 levels were increased in patients with early RA and established RA compared to healthy controls (41). Thus, we tested the mRNA level of these 2 associated genes. MMP3 and caspase-1 mRNA expression was downregulated in the CIA ankle tissues of anti-CHI3L1 Ab-treated mice (**Fig. 5B**). To confirm the relationship between CHI3L1, MMP3 (and other MMPs), and caspase-1, we analyzed the expression of MMP3, MMP9, MMP13, and caspase-1 in ankle tissues via western blotting. Anti-CHI3L1 Ab treatment reduced the expression of MMP3, MMP9, MMP13, and caspase-1 in CIA mice (**Fig. 5C**). Anti-TNF-α Ab therapy also reduced the expression of these proteins, but the effect was weaker than with anti-CHI3L1 Abs (**Fig. 5C**). We further investigated whether CHI3L1 was involved in the regulation of MMP3, MMP9, MMP13, and caspase-1 in THP-1 and MH7A cells. Similar to the results in the CIA model, treatment with anti-CHI3L1 Ab reduced the expression of

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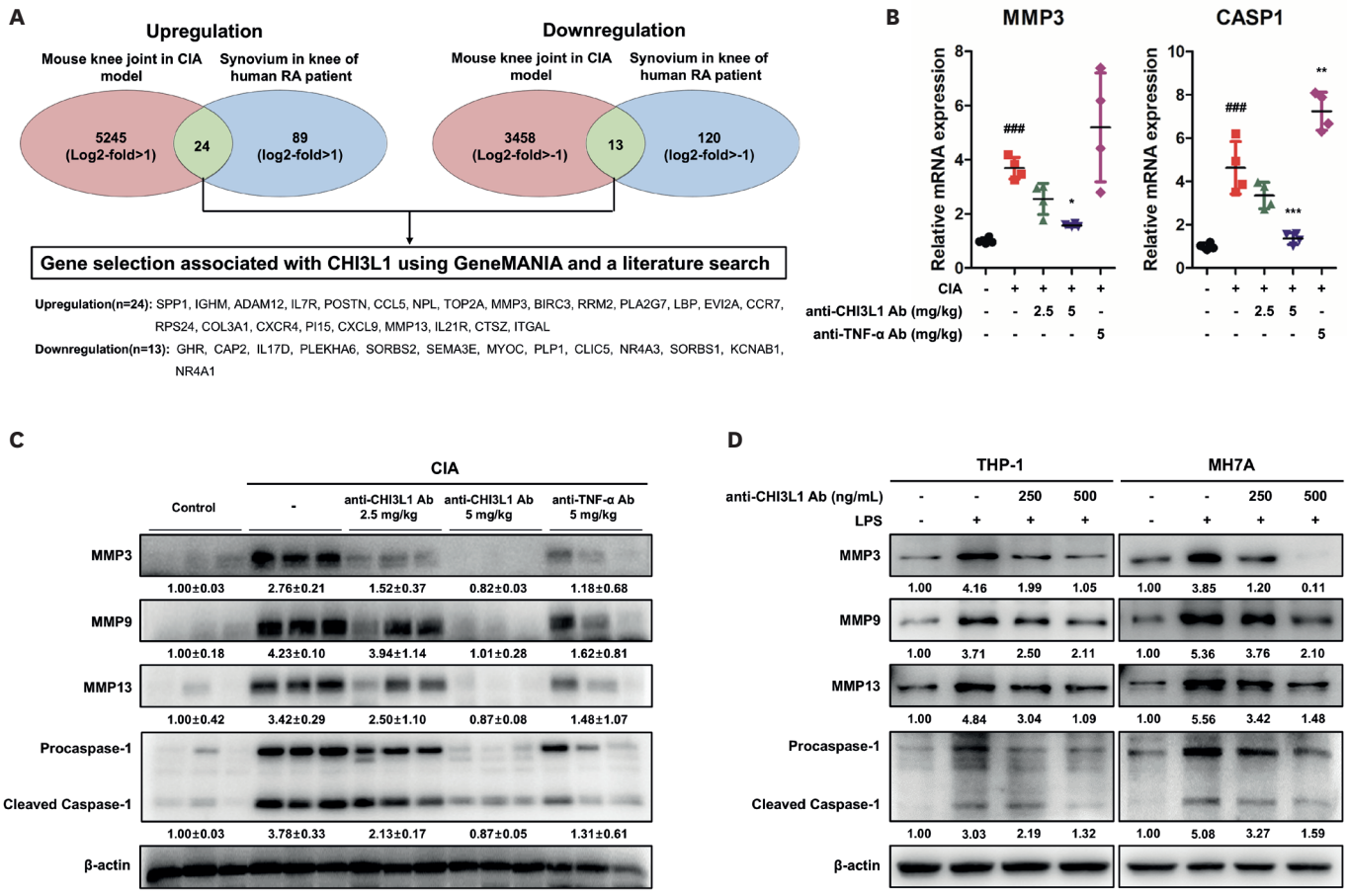

Figure 5. MMP3 is involved in CHI3L1-mediated RA development. (A) Target gene selection strategy. Co-upregulated or co-downregulated genes from RNA sequencing analysis of knee joint of mouse CIA mode and human knee synovium of RA patients. (B) mRNA expression of MMP3 and caspase-1 in mouse CIA ankle tissue samples by qPCR. (C) Expression of MMP3, MMP9, MMP13 and caspase-1 in mouse CIA ankle tissue samples by Western blot analysis. (D) PMA-treated THP-1 and MH7A cells were pretreated with anti-CHI3L1 Ab for 1 h. Then, cells were stimulated with LPS (100 ng/ml) for another 24 h. Expression of MMP3, MMP9, MMP13 and caspase-1 were analyzed by Western blot analysis.
Non-CIA control vs. CIA control: ###p<0.001.
CIA control vs. CIA treated with anti-CHI3L1 Ab or anti-TNF-α Ab: *p<0.05; **p<0.01; ***p<0.001.

MMP3, MMP9, MMP13, and caspase-1 in LPS-treated THP-1 and MH7A cells (**Fig. 5D**). These data suggest that potential effectiveness of anti-CHI3L1Ab in arthritis treatment could be associated with MMP3 expression.

MMP3 knockdown reduces inflammation *in vitro*

As shown in previous results (42), targeting CHI3L1 suppresses the expression of MMPs and caspase-1 under inflammatory conditions. Because MMP3 showed the highest inhibitory activity in the mouse CIA model and LPS-induced THP-1 and MH7A cells following anti-CHI3L1 Ab treatment, we first determined whether MMP3 affected the expression of other MMPs and caspase-1. The siRNA-mediated inhibition of MMP3 suppressed the expression of MMP9, MMP13, and caspase-1 in THP-1 and MH7A cells with or without LPS stimulation (**Fig. 6A and B**). We also found that MMP3 knockdown reduced the levels of RA-related cytokines, including TNF-α, IL-1β, IL-18, and CCL2 mRNA and TNF-α level in THP-1 and MH7A cells with or without LPS stimulation (**Fig. 6C and D**).

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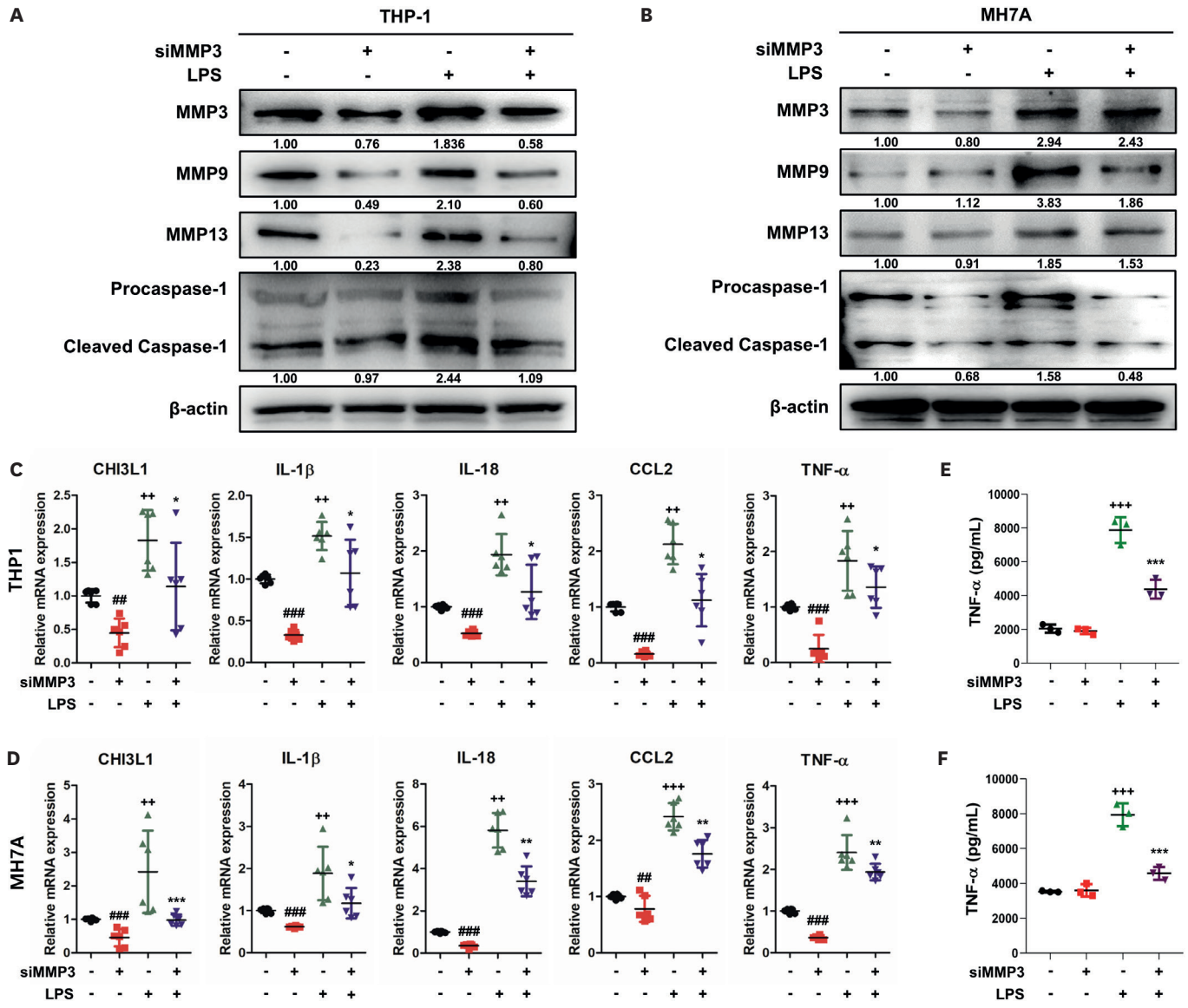

Figure 6. Effect of MMP3 knockdown on LPS-induced inflammation in THP-1 and MH7A cells. PMA-treated THP-1 and MH7A cells were transfected with MMP3 siRNA (20 nM). After 24 h, cells were stimulated with LPS (100 ng/ml) for another 24 h. (A, B) Expression of MMP3, MMP9, MMP13 and caspase-1 were detected by Western blot analysis. (C, D) The mRNA levels of CHI3L1, IL-1β, IL-18, CCL2 and TNF-α were analyzed by qPCR. (E, F) Levels of TNF-α in *in vitro* supernatant samples were analyzed by ELISA (n=3).
Control vs. siMMP3: $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$.
Control vs. LPS: $^{++}p<0.01$; $^{+++}p<0.001$.
LPS vs. LPS with siMMP3: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

DISCUSSION

RA is an autoimmune disease characterized by synovial inflammation, cartilage damage, and bone erosion (1). Research conducted for several decades have demonstrated a clear correlation between CHI3L1 and the development of RA, and previous studies have shown the efficacy effects of anti-CHI3L1 Abs on inflammation-related disease models such as phthalic anhydride-induced atopic disease and LPS-induced acute liver injury models (8-11). Therefore, we sought to determine whether a CHI3L1-targeting Ab is also effective in the treatment of RA. The serum and synovial fluid levels of CHI3L1 were higher and related

to disease severity in patients with RA than in normal controls (7,43,44). In the present study, we also found that serum CHI3L1 levels were significantly higher in patients with RA than in healthy controls. ROC curve analysis indicated that the diagnostic performances of serum CHI3L1, anti-CCP, and CRP were comparable. Spearman's correlation coefficient indicated that serum CHI3L1 and RF were significantly correlated in patients with RA. In addition, a meta-analysis confirmed the clinical correlation between CHI3L1 and RA (7). Our previous studies demonstrated that CHI3L1-targeted therapy using chemical inhibitors and mAbs alleviated atopic dermatitis and neurological diseases by inhibiting inflammation (8-10,45). In addition, PGE_2 and NO are 2 pleiotropic inflammatory mediators overproduced in arthritis, and COX2 and iNOS are overexpressed in both cartilage and synovial in the RA tissues (46). And CHI3L1 could control iNOS and COX-2 expression in the inflammatory responses of cells (8,45). Our results demonstrate that targeting CHI3L1 decreased the expression of inflammation-related factors COX-2 and iNOS, and reduced PGE_2 and NO levels, which are elevated in arthritis. In this regard, this study also showed that anti-CHI3L1 Ab administration reduced inflammatory cell infiltration and the expression of RA-related cytokines, such as TNF-α, IL-1β, IL-18, and CCL2-expression in ankle tissue samples of a mouse CIA model. In addition, CHI3L1 blockade reduced the levels of TNF-α, IL-1β, IL-18, and CCL2 as well as inflammatory mediators; nitric oxide and PGE_2 release in LPS-treated THP-1 and MH7A cells. These results suggested that the suppression of CHI3L1 may be critical for preventing RA development by reducing the levels of inflammatory mediators and that anti-CHI3L1 Ab could be a new biologic anti-rheumatic drug. However, the exact mechanism by which anti-CHI3L1 Abs inhibit RA development remains unclear.

MMPs are key enzymes that degrade extracellular matrix components, playing a critical role in joint damage observed in RA (47). Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are known to stimulate MMP production, thereby driving cartilage destruction (48). Interestingly, recent studies have demonstrated a significant correlation between CHI3L1 levels and both MMP3 and IL-6 levels in the serum of patients with RA and osteoarthritis (49,50). To investigate key target genes in RA, we utilized publicly available microarray data from RA patients and mouse CIA models. Our analysis identified MMP3 as a highly relevant target in RA pathogenesis. This finding was further supported by GeneMANIA analysis, which demonstrated a strong association between CHI3L1 and MMP3. Importantly, MMP3 expression has been observed in various cell types, including synovial cells, chondrocytes, and macrophages (13).

Previous studies have shown that MMP3 expression is regulated by several signaling pathways. It is reported that the inhibition of JNK signaling reduced MMP3 expression and IL-6 production in RA fibroblast-like synoviocytes (51). Also, TNF-α mediated activation of p38 MAPK signaling induced MMP3 production in MH7A synoviocytes (52). Another study revealed that NF-κB p50 and p65 bind to the MMP3 promoter in THP-1 cells (53). Further, IL-1β-induced activation of ERK1/2 and the PI3K/Akt lead to increase MMP3 expression in mouse primary chondrocytes (54). In our previous studies showed that CHI3L1 regulates NF-κB and MAPK activation in experimental disease models of atopic dermatitis, AD and lung cancer (10,42,45). Based on these findings, anti-CHI3L1 Ab therapy could prevent cartilage damage by blocking the increase in MMP3 expression mediated through NF-κB and MAPK signaling.

In fact, in this study, we observed that recombinant CHI3L1 treatment significantly increased the secretion of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in THP-1 and MH7A cells. Conversely, treatment with anti-CHI3L1 Ab significantly decreased the levels of these cytokines. Furthermore, anti-CHI3L1 Ab treatment effectively reduced the expression of

MMP3, MMP9, and MMP13 to basal levels in both the mouse CIA model and in LPS-stimulated THP-1 and MH7A cells. Additionally, knockdown of MMP3 resulted in a significant decrease in the expression of MMP3 itself, caspase-1, and pro-inflammatory cytokines, including TNF-α, IL-1β, IL-18, and CCL2, at both the protein and mRNA levels in THP-1 and MH7A cells. These findings strongly suggest that anti-CHI3L1 Ab therapy may effectively prevent articular damage by inhibiting MMP3-mediated production of pro-inflammatory cytokines.

Pro-IL-1β and pro-IL-18 are cleaved into their mature, biologically active forms by caspase-1 (55). Studies in caspase-1-deficient mice have demonstrated reduced synovial inflammation, cartilage damage, and bone erosion in a model of streptococcal cell wall fragment-induced chronic arthritis (40). Furthermore, a clinical study revealed elevated levels of active caspase-1 in patients with both early and established RA compared to healthy controls (41). Our analysis of publicly available microarray data from RA patients and mouse CIA models identified caspase-1, in addition to MMP3, as a potential target for RA therapy. Consistent with this, treatment with anti-CHI3L1 Ab in the mouse CIA model and in LPS-stimulated THP-1 and MH7A cells significantly decreased the protein expression of both caspase-1 and MMP3. Moreover, knockdown of MMP3 in these cell lines led to a significant decrease in LPS-induced caspase-1 expression, accompanied by reduced expression of other MMPs and decreased mRNA levels of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-18, and CCL2. These findings suggest that the inhibitory effects of anti-CHI3L1 Abs on MMP3-mediated inflammatory responses and RA development may involve the suppression of caspase-1 activation. However, the precise mechanisms underlying the regulation of caspase-1 by MMP3 remain to be fully elucidated. While the regulatory relationship between MMP3 and caspase-1 is complex, previous studies have demonstrated reciprocal regulatory interactions. For example, MMP3 deficiency has been shown to reduce caspase-1-mediated inflammatory responses in the retina and retinal pigment epithelium of LPS-treated mice (56). Conversely, inhibition of caspase-1 activity suppresses the expression of MMPs, including MMP3, in cerebral infarction (57). Additionally, recombinant IL-1β treatment has been shown to upregulate MMP3-dependent caspase-1 expression in chondrocytes and synoviocytes (58,59). Based on these findings, we hypothesize that the preventive effects of anti-CHI3L1 Abs on RA development may be mediated, at least in part, by the inhibition of both MMP3 and caspase-1.

An imbalance in M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage populations is well-established as a significant contributor to the pathogenesis of RA (38). In this study, we observed that anti-CHI3L1 Ab treatment inhibited the infiltration and/or polarization of both M1 and M2 macrophages within the joints of CIA mice. Previous studies have demonstrated a role for CHI3L1 in macrophage polarization. Blockade of CHI3L1 has been shown to reduce M1 microglia differentiation in neuroinflammation within the Tg2576 mouse model (45). Furthermore, CHI3L1 is predominantly induced in macrophages during Th2-dependent skin inflammation (60). Consistent with our findings, it has been reported that CHI3L1-deficient mice exhibit suppressed Th2 immune responses and a shift towards M2 macrophage polarization in the context of food allergy development (61). Our previous research also demonstrated that CHI3L1 depletion decreases M2 macrophage polarization, which is potentially associated with the alleviation of liver sepsis in CHI3L1-deficient mice (11). Importantly, inhibition of M1 macrophage polarization has been shown to reduce MMP3 expression and pro-inflammatory responses within the joints of CIA-induced animal models (62,63). Therefore, modulation of macrophage polarization, particularly the inhibition of pro-inflammatory M1 macrophage infiltration and activation, may represent a crucial mechanism underlying the inhibitory effects of anti-CHI3L1 Abs on CIA-induced pro-inflammatory responses and RA development.

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The majority of FDA-approved RA therapies are anti-inflammatory agents, including corticosteroids, JAK inhibitors, TNF inhibitors, IL-6R inhibitors, and anti-CD20 Abs (64). Among these, anti-TNF-α Abs, such as adalimumab and infliximab, are widely used and generate significant global revenue. While not directly compared in this study, anti-CHI3L1 Ab treatment demonstrated potential advantages over anti-TNF-α therapy. Specifically, in our preclinical models, anti-CHI3L1 Abs exhibited a more rapid onset of clinical improvement and a greater reduction in MMP expression and inflammatory cytokine levels compared to anti-TNF-α Abs at comparable doses. Previous cohort studies have indicated that patients with RF-positive RA may exhibit a poorer therapeutic response to anti-TNF-α agents compared to RF-negative patients (65,66). Our findings further support this observation, demonstrating a significant association between serum CHI3L1 and RF levels in RA patients. Conversely, a clinical study demonstrated a correlation between CHI3L1 levels and disease activity (as measured by the DAS28 score) in DMARD-naïve patients with early RA undergoing conventional synthetic DMARD therapy with infliximab (49). Based on these findings, anti-CHI3L1 Ab therapy may offer a promising therapeutic approach for RA, potentially overcoming the limitations observed with current therapies, such as suboptimal responses in certain patient populations.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Information for Abs and kits

Supplementary Table 2
Primer sequence information

Supplementary Table 3
The list of up-regulated and down-regulated genes in both human RA patients and mouse CIA model

Supplementary Figure 1
The effects of anti-CHI3L1 Ab on the activation of the inflammation signaling in the tissues of CIA mice. Development of rheumatic arthritis in the CIA mice was monitored for 24 days. (A, B) IHC analysis was used to evaluate the expression of F4/80, Ly6G, iNOS and MRC1 in the joints, (C, D) and the mRNA and protein expression levels of M1 macrophage markers (iNOS and CD86) and M2 macrophage markers (MRC1 and ARG1) were measured by qPCR and Western blotting.

Supplementary Figure 2
Correlation between CHI3L1 with IL-7R, NPL, MMP3, LBP, COL3A1 and CXCL9 using GeneMANIA.

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