Chimeric anti-IL-17 full-length monoclonal antibody is a novel potential candidate for the treatment of rheumatoid arthritis

FULIANG BAI, HUI TIAN, ZESHAN NIU, MINGYAO LIU, GUIPING REN, YINHANG YU, TIAN SUN, SIMING LI and DESHAN LI

Biopharmaceutical Teaching and Research Section, College of Life Science, Northeast Agricultural University, Harbin, Heilongjiang 150030, P.R. China

Received July 30, 2013; Accepted November 4, 2013

DOI: 10.3892/ijmm.2013.1611

Abstract. Rheumatoid arthritis (RA) is an autoimmune disease, primarily manifesting as inflammatory arthritis. It is associated with chronic inflammation of the synovial joints, mostly in the hands and feet, as well as with systemic extra-articular inflammation. The chimeric anti-interleukin (IL)-17 full-length monoclonal antibody (CMa17Aab) targets IL-17A, which is an important cytokine in the pathogenesis of RA and other inflammatory disorders. In this study, we investigated whether CMa17Aab exerts therapeutic effects in a mouse model of type II collagen-induced arthritis (CIA). Mice with CIA were subcutaneously injected with the humanized CMa17Aab antibody. The effects of treatment were assessed by estimating the arthritis severity score, the extent of histological damage and bone destruction, the autoreactive humoral and cellular immune responses and the production of cytokines. Treatment with CMa17Aab exerted beneficial effects in the mice with CIA as regards clinical and histological parameters. Compared with the controls, treatment with CMa17Aab resulted in a significant alleviation of the severity of the symptoms of arthritis, by preventing bone damage and cartilage destruction, reducing humoral and cellular immune responses, and downregulating the expression of IL-6, IL-8, matrix metalloproteinase (MMP)-3, IL-17, IL-1β, tumor necrosis factor (TNF)-α, receptor activator for nuclear factor-κB ligand (RANKL) and interferon (IFN)-γ in inflamed tissues. In conclusion, our study demonstrates that treatment with CMa17Aab exerts beneficial effects in mice with CIA, by preventing joint inflammation, cartilage destruction and bone damage. These preliminary results suggest that CMa17Aab is an important regulator in RA, and that it may represent a novel therapeutic agent that may prove useful in the treatment of this disease.

Correspondence to: Professor Deshan Li, Biopharmaceutical Teaching and Research Department, College of Life Science, Northeast Agricultural University, 59 Mucai Street, Box 180, Harbin, Heilongjiang 150030, P.R. China
E-mail: deshanli@163.com

Key words: rheumatoid arthritis, collagen-induced arthritis, CMa17Aab

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by chronic inflammation, mainly of the synovial joints, which affects numerous organs and systems, and may lead to cartilage destruction and deformation, resulting in chronic pain, severe disability and increased mortality rates (1). Although the causes of RA are not yet fully understood, laboratory and clinical evidence suggests that pro-inflammatory cytokines, particularly interleukin (IL)-17, play an important role in the pathogenesis of this disease (2-5). IL-17A is a T cell-derived pro-inflammatory cytokine produced in the rheumatoid synovium. Some RA synovial T cells producing IL-17 can activate mesenchymal cells, leading to an increased pro-inflammatory pattern sensitive to regulation by Th2-type cytokines. IL-17, particularly when combined with tumor necrosis factor (TNF)-α, may contribute to the progression of RA, notably through their combined effect on synoviocyte aggressiveness (6,7). In patients with RA, high local levels of IL-17 are detected in both the synovium and synovial fluid (8,9). IL-17 induces the production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) (10,11) and CXC chemokines, which recruit neutrophils into the joints (12,13), stimulate angiogenesis (14,15), and are implicated in joint degradation (16). The relevance of IL-17 for RA has been further highlighted by *in vitro* studies performed in the presence of TNF-α and IL-1β, two key pathogenic pro-inflammatory cytokines overexpressed in the joints of patients with RA (17,18). IL-17 synergistically acts with these two cytokines to induce inflammatory mediators in synoviocytes, human osteoblasts, myoblasts and chondrocytes (19-21). Furthermore, a clinical study demonstrated that synovial IL-17 overexpression is associated with damage to the joints (22).

Although IL-17A shows affinity to the IL-17 receptor A (IL-17RA), binding assays have suggested that it can bind additional receptors. To date, four additional receptors have been identified in the IL-17R family based on sequence homology to IL-17R (IL-17Rh1, IL-17RC, IL-17RD and IL-17RE) (23). Functional IL-17R is a heteromeric complex of IL-17RA and IL-17RC proteins (24). A number of different components of the complex exist, including IL-17RB (25), which binds preferentially to IL-17B and IL-17E (26,27). IL-17RA and IL-17RC have been found to be overexpressed in RA peripheral whole blood cells and their expression has been detected in the

synovium of patients with RA (28). Their levels are increased in serum and synovial fluid in patients with RA and with other autoimmune and inflammatory conditions.

In inflammatory arthritis, IL-17 plays a key role in the propagation of joint inflammation, cartilage destruction and bone erosion (29). In recent years, the use of anti-IL-17 antibodies in RA therapy has been investigated by an increasing number of researchers. Lubberts *et al* (30) reported that treatment with a neutralizing anti-murine IL-17 antibody after the onset of collagen-induced arthritis (CIA) reduced joint inflammation, cartilage destruction and bone erosion. Genovese *et al* (31) reported that a humanized anti-IL-17 monoclonal antibody is an efficient anti-rheumatic agent, in a clinical trial in patients with RA. Nardelli *et al* (32) demonstrated that the treatment of interferon (IFN)-γ-deficient mice vaccinated and challenged with *Borrelia burgdorferi* with a murine anti-IL-17 antibody exerted therapeutic effects against severe destructive arthritis. Kotake *et al* (9) demonstrated that the levels of IL-17 were significantly higher in the synovial fluid of patients with RA compared with patients with osteoarthritis and that a murine anti-IL-17 antibody significantly inhibited osteoclast formation. Although numerous studies have suggested that anti-IL-17 antibodies alleviate the symptoms of RA, a considerable number of improvements in this type of therapy are required with regards to safety and immunomodulatory effects. In this study, we investigated whether the recombinant chimeric anti-IL-17 full-length monoclonal antibody (CMa17Aab) exerts therapeutic effects by neutralizing IL-17. DNA encoding the murine anti-human IL-17 was linked to DNA encoding the Fc portion of a human IgG1 molecule, and the combined DNA was then expressed in a mammalian cell line. CMa17Aab may be used to reduce anti-xenogeneic immunoglobulin response and anti-idiotypic response by employing alternative portions of the murine Fc fragment. Differences in the constant region of the heavy chain of CMa17Aab determine the immunoglobulin isotypes, which can alter the effects of the antibody *in vivo*, such as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and immunomodulatory effects. Our results demonstrate that CMa17Aab acts as a competitive inhibitor of IL-17 and prevents the binding of IL-17 to the cell surface receptor, IL-17R, thereby reducing the biological activity of IL-17. No adverse effects were detected following the intravenous administration of CMa17Aab in DBA/1J mice. There were trends toward a reduction in disease activity in a safety and dose-finding study of CMa17Aab administered for four weeks to a small number of mice with refractory RA. Based on these findings, we conclude that CMa17Aab may be an ideal therapeutic agent for clinical application in patients with active, refractory RA.

Materials and methods

Cell lines and reagents. CHO-K1SV and HeLa cells were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). RA synoviocytes were kindly provided by Harbin Medical University. The CHO-K1SV cell line was cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) (both from Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS. RA synoviocytes were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin, at 37˚C in a humidified 5% CO_2 incubator. Restriction enzymes and the DNA ligation and purification systems were purchased from New England Biolabs (Hitchin, UK).

Construction of expression vectors and stable expression of CMa17Aab in CHO-K1SV cells. The construction of the neutralizing mouse anti-human IL-17 monoclonal antibody was confirmed by fluorescence-activated cell sorting (FACS). The single chain variable fragments (ScFvs) of the mouse anti-human IL-17 monoclonal antibody were cloned and inserted into the Pklight vector (termed Pklight-anti-IL-17 vector). The constant domain heavy chain (CH)-Fc and light chain (CL) fragments were subcloned into the Pklight-anti-IL-17 vector, subsequently termed Pklight-CMa17Aab. Pklight-CMa17Aab and IREX-enhanced green fluorescent protein (EGFP) were subcloned into the Peedual12.4 vector (termed Peedual12.4-CMa17Aab vector) which contained the glutamine synthetase (GS) gene. Peedual12.4-CMa17Aab was transfected into the CHO-K1SV cells using Lipofectamine 2000. Thus, CMa17Aab and EGFP were both stably expressed in the CHO-K1SV cells. The stable and high expression of CMa17Aab in the CHO-K1SV cells was confirmed using a flow cytometer (BD FACSAria™ Cell Sorter; BD Biosciences, Franklin Lakes, NJ, USA).

Purification of CMa17Aab from the supernatant of CHO-K1SV cells. The supernatant of serum-free CHO-K1SV cells was harvested. CMa17Aab was purified using protein-A affinity and size-exclusion chromatography selecting for purity of the monomer >90%.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified CMa17Aab fragment was resolved by 12% SDS-PAGE under reducing conditions with β-mercaptoethanol (β-ME).

ELISA. In the cross-linking assay, CMa17Aab was examined for its capacity to simultaneously bind two target antigens: hIL-17 and mIL-17. Purified CMa17Aab was determined by ELISA. CMa17Aab or anti-IL-17 antibody (ScFv) (0.2-1.0 mg/ml) were added into a 96-well microplate and incubated at 4˚C for 12 h. hIL-17 or mIL-17 (2 μg/ml) were then added to the wells and incubated for 30 min at 37˚C, with CMa17Aab replaced by BSA in the controls. After five washes with PBST [20 mmol/l phosphatase-buffered saline (PBS), 0.05% Tween-20], 100 μl mouse HRP-conjugated anti-hIL-17 monoclonal antibody (eBioscience, Inc., San Diego, CA, USA) was added followed by incubation for 1 h. Following five washes with PBST, the DAB/H_2O_2 system was used for detection. The results were recorded at 450 nm on an ELISA plate reader.

ADCC and CDC assays. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation in a Ficoll-Hypaque density gradient. ADCC and CDC of CMa17Aab were measured by lactate dehydrogenase (LDH) assay (Merck KGaA, Darmstadt, Germany), which measures the activity of

LDH released from the cytosol of damaged cells. HeLa cells stably expressing transmembrane IL-17A were incubated with various concentrations of CMa17Aab for 1 h in assay medium (DMEM + 5% FBS) in a 5% CO_2 incubator at 37˚C, followed by the addition of either human PBMCs as effector cells (effector to target ratio, 20:1 for the ADCC assay) or human complement serum (Quidel Corp., San Diego, CA, USA) (1.25% vol/vol for the CDC assay). Following an additional incubation at 37˚C for 16 h for the ADCC assay and 5 h for the CDC assay, 100 μl of supernatant from each well were transferred to a clean flat-bottom 96-well plate. LDH substrate (100 μl) was added to each well followed by incubation for 30 min at room temperature in the dark. The absorbance of the samples was measured at 490 nm on an ELISA plate reader.

RNA extraction and real-time RT-PCR. Total RNA (1 μg) was reverse transcribed using the ThermoScript RT-PCR System (Invitrogen Life Technologies). The concentration of RNA was quantified by spectrophotometry at 260 nm (SmartSpec™ 3000; Bio-Rad, Hercules, CA, USA). Briefly, total RNA was denatured by incubation for 5 min at 70˚C with 4 μM oligo(dT) primers and then reverse transcribed using 0.5 mM dNTP, 40 U/μl RNaseOUT, 0.01 M DTT and 10 U/μl of the ThermoScript Reverse Transcriptase enzyme (final concentrations). Reverse transcription was performed by incubation at 42˚C for 180 min. The obtained cDNA was diluted 1/10 with distilled water and 10 μl of the dilution were used for amplification. Specific primer sets for IL-6, IL-8, matrix metalloproteinase (MMP)-3, IL-17, IL-1β, TNF-α, receptor activator for nuclear factor-κB ligand (RANKL) and IFN-γ were conserved in our laboratory. Primer sets for IL-17RA (GenBank accession no. NM_014339) were synthesized by Invitrogen as follows: IL-17RA forward, 5'-AGACACTCCAGAACCAATTCC-3' and reverse, 5'-TCTTAGAGTTGCTCTCCACCA-3'. PCR was performed using the LightCycler FastStart DNA SYBR-Green I kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's instructions on the parameters: 45 amplification cycles, denaturation at 96˚C, primer annealing at 68˚C with touchdown at 58˚C, and amplicon extension at 72˚C.

Animal experiments. To establish the mouse model of CIA, 8-10-week-old DBA/1J mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were kept in a temperature-controlled environment in a 12-h light/dark cycle. The induction of type II CIA was achieved as previously described (33) by the subcutaneous injection of 2, 4 or 8 mg collagen (ModiQuest Research, Oss, The Netherlands) per mouse (n=9 in each group) (34). Clinical arthritis scores were evaluated using a scale of 0-2 for each paw for a total score of 8. Paws were assigned a clinical core based on the following scoring method (ModiQuest Research): 0, normal; 0.25, one or two swollen toes; 0.5, three to four swollen toes; 0.75, slightly swollen footpad or ankle; 1, swollen footpad or ankle; 1.25, one or two swollen toes and swollen footpad or ankle; 2.0, swollen toes, footpad and ankle. Treatment commenced on day 21 after the initial immunization. Mice were administered 10 mg/kg CMa17Aab by subcutaneous injection for nine consecutive days and were sacrificed on day 70.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Chinese Association For Laboratory Animal Sciences (CALAS), Animal Health Products, Committee on the Ethics of Animal Experiments Defence Research and Development, China and Animal Experiments of the University of Northeast Agricultural (approval number: SCXK-2012-0002). All surgical procedures and euthanasia were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Statistical analysis. Statistical significance was determined with a two-tailed Student's t-test using Excel software. One-way ANOVA, the Mann-Whitney U test, Kaplan-Meier and log-rank statistical analyses were performed using MedCalc software (MedCalc, Ostend, Belgium).

Results

Construction of vector and stable expression of the recombinant anti-human IL-17A high-affinity antibody in CHO-K1SV cells. The recombinant vector Peedual12.4, expressing CMa17Aab was constructed as described in Materials and methods (Fig. 1A). The resulting vector containing EGFP and GS genes, was termed Peedual-12.4-CMa17Aab. The expression of CMa17Aab in the CHO-K1SV cells was determined by screening with EGFP using FACS, a simple, rapid, sensitive and reliable method. The expression of CMa17Aab in the CHO-K1SV cells was also determined by screening with GS, using the methionine sulfoximine (MSX) system. A screening system using EGFP and GS has the advantage of emitting fluorescence without the addition of any substrates. The system was excitated by a 488-nm laser and the emission was detected through a 530/30 band-pass filter. For each sample, 10,000 or 20,000 populations of cells were analyzed, and cells with a higher fluorescence intensity as compared with the background-defined threshold (from cells not transfected with the Peedual-12.4-CMa17Aab vector) were calculated. The selected cells were plated into 96-well culture plates, and subcloned one month later. The results revealed that a polyclone with a stable and high expression of CMa17Aab was successfully obtained.

CMa17Aab blocks the gene expression of IL-6 and IL-8 stimulated by IL-17A. The supernatant of the CHO-K1SV serum-free cells was harvested after three days of culture and the CMa17Aab antibody was purified using protein-A affinity and size-exclusion chromatography. Purified CMa17Aab was analyzed on a 12% SDS-PAGE gel (Fig. 2A), which confirmed purification. DNA and endotoxins were removed from purified CMa17Aab for the subsequent animal experiments. To evaluate the binding specifity of purified CMa17Aab, a series of ELISAs were performed. Binding to IL-17 was assessed using an anti-IL-17 scFv antibody as the positive control. As shown in Fig. 2B, the results revealed the binding of CMa17Aab to mIL-17 and hIL-17. We also evaluated the ability of CMa17Aab to block the binding of hIL-17A with IL-17RA. hIL-17A (at a final concentration of 100 ng/ml) was added to the HeLa cells, the cells were cultured at 37˚C for 12 h, and the gene expression of IL-6 and IL-8 was measured by real-time RT-PCR. The hIL-17A-stimulated cells showed an increase in the gene expression of IL-6 (CMa17Aab-treated cells and untreated cells vs. IL-17-treated cells, P=0.0036 and

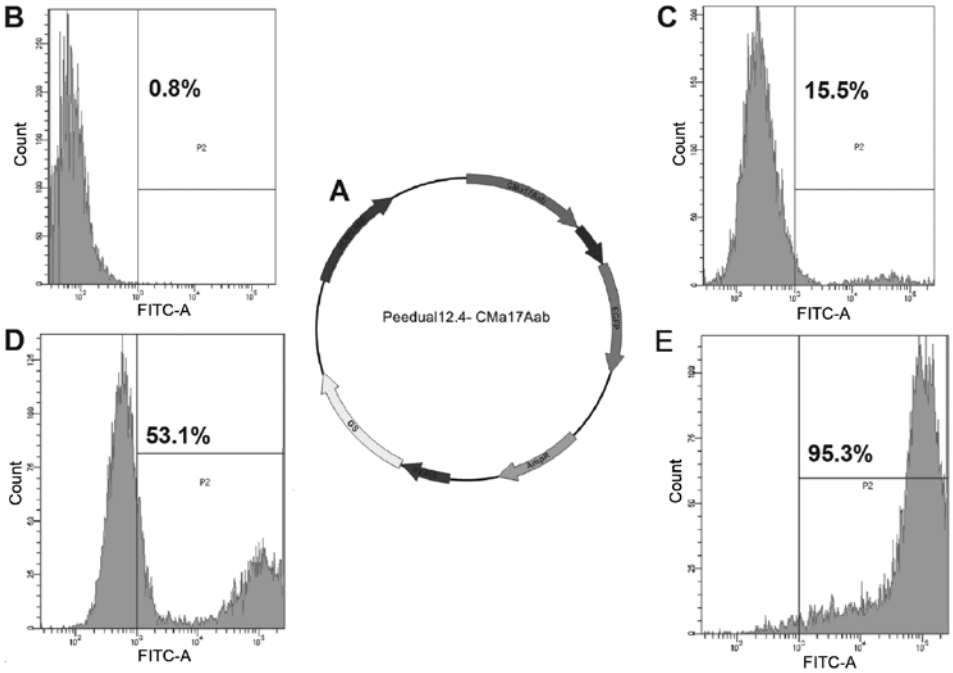

Figure 1. Construction of the CMa17Aab vector, and stable and high expression of CMa17Aab in CHO-K1SV cells as screened by FACS. (A) The chimeric anti-interleukin (IL)-17 full-length monoclonal antibody was inserted into the Peedual-12.4 vector along with EGFP. The GS gene, also inserted, provided an additional means of detecting the antibody using cell sorting in Iscove's modified Dulbecco's medium (IMDM) with methionine sulfoximine (MSX). (B-E) Fluorescence detection and estimated positive rates. CHO-K1SV cells were washed and resuspended in phosphate-buffered saline (PBS) twice. The cells were identified and sorted in a flow cytometer at 488 nm. (B) Control; (C) CHO-K1SV cells transfected with Peedual-12.4-CMa17Aab, 15.5% positive rate; (D) Screening of the P2 area of B, 53.1% positive rate; (E) Screening of the P2 of C, 95.3% positive rate.

P=0.0024, respectively) and IL-8 (CMa17Aab-treated cells and untreated cells vs. IL-17-treated cells, P=0.0075 and P=0.0078, respectively) (Fig. 2D and E). Student's paired two-tailed t-tests revealed that these differences were statistically significant (P<0.05, P<0.01 vs. IL-17-treated cells).

IL-17 detection in peripheral whole blood and IL-17R overexpression in synovial tissue from mice with CIA. To determine whether IL-17 and IL-17R are differentially expressed in mice with CIA as compared with healthy mice, we examined IL-17 expression at the mRNA level in whole blood samples by real-time RT-PCR (Fig. 3A). A significant upregulation of IL-17 expression in peripheral whole blood was observed in the mice with CIA (n=9) as compared with the healthy mice (n=3). This difference mainly reflects the overexpression of IL-17 in mice with severe CIA (healthy mice vs. mice with primary, moderate and severe CIA: 4.9-, 10.22- and 29.09-fold, respectively; P<0.05, P<0.01 and P<0.01, respectively). These results revealed that IL-17 mRNA expression was higher in the mice with primary, moderate and severe CIA mice as compared with the healthy mice.

As it has been previously suggested that IL-17 acts as a heterodimer to transduce IL-17R signals (35), we examined the expression of IL-17R in synovial tissue (n=9). We demonstrated a correlation between the mRNA expression of IL-17R and the development of illness in the synovial tissue of mice with CIA (primary, moderate and severe CIA: P<0.05, P<0.01 and P<0.01, respectively). Moreover, it should be noted that the IL-17R mRNA levels in mice with CIA were higher than those in the healthy mice (Fig. 3B).

Ability of CMa17Aab to mediate ADCC and CDC. Previous studies have reported that the binding of antibodies to the free form of IL-17 is important in the treatment of RA, since it can lead to the suppression of cell surface-expressed IL-17A through ADCC or CDC (30,36,37); this may be one of the reasons responsible for the differential effects observed during the clinical treatment of diseases with anti-IL-17 antibodies. The cell surface expression of IL-17RA on macrophages and monocytes plays a critical role in granulomatous diseases such as Crohn's disease and Wegener's granulomatosis, and the cells can be directly killed through ADCC or CDC. When CMa17Aab binds to IL-17A following IL-17-antagonist binding to cells expressing the transmembrane form of IL-17RA, these cells are targeted by natural killer cells, triggering systemic complement activation. The presence of the Fc region of human IgG1 in CMa17Aab may induce cell lysis in IL-17RA-producing cells. The ability of CMa17Aab to mediate ADCC and CDC in cells expressing the transmembrane form of IL-17R was examined in this study. In the ADCC assay, >20% of the IL-17RA-bearing HeLa target cells were lysed by CMa17Aab at 6.25 mg/ml at a 20:1 effector to target ratio (Fig. 4A). In the CDC assay, CMa17Aab induced the lysis of transmembrane IL-17RA cells in the presence of human complement serum (Fig. 4B). These data indicate that CMa17Aab mediates ADCC and CDC upon binding to transmembrane IL-17RA expressed on the cell surface and therefore, there is considerable potential to develop CMa17Aab into a more effective IL-17A-neutralizing antibody, similar to other therapeutic antibodies already in use with the ability to induce ADCC and CDC.

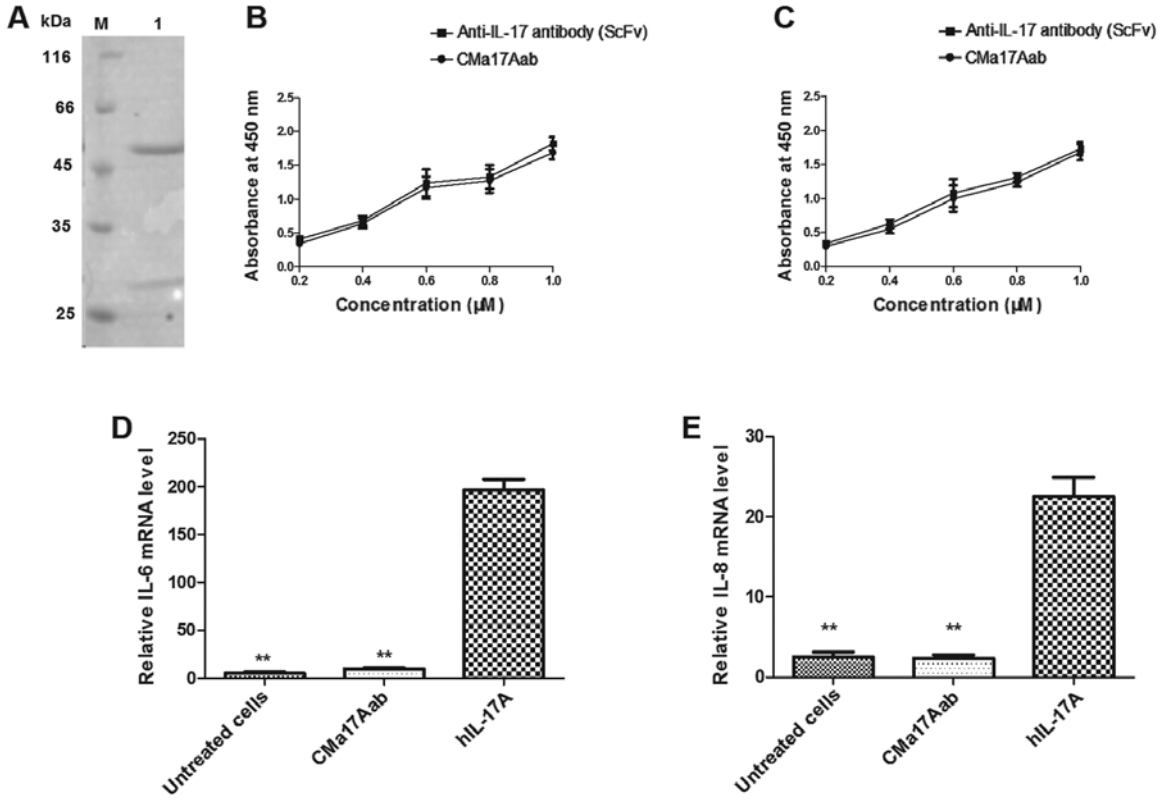

Figure 2. SDS-PAGE, ELISA and biological activity of purified CMa17Aab. (A) 12% SDS/Bis-Tris-PAGE of CMa17Aab (lane 1) under reducing conditions. CMa17Aab was expressed and purified from CHO-K1-SV cells. The gel was stained with Coomassie blue. M, molecular mass standard. (B and C) ELISA analysis of the binding affinity of CMa17Aab to hIL-17 and mIL-17. (D and E) Gene expression of interleukin (IL)-6 and -8, as measured by real-time RT-PCR. The untreated cells were used as the control. Expression of each gene was calculated relative to the expression of the housekeeping gene, β-actin. Data represent the means ± standard error of the mean (SEM) of triplicate samples. CMa17Aab-treated cells and untreated cells vs. the hIL-17A antigen group of IL-6/IL-8: P=0.0036, P=0.0024/P=0.0075, P=0.0078, respectively. *P<0.05 and **P<0.01, vs. IL-17-treated cells from Student's paired two-tailed t-tests.

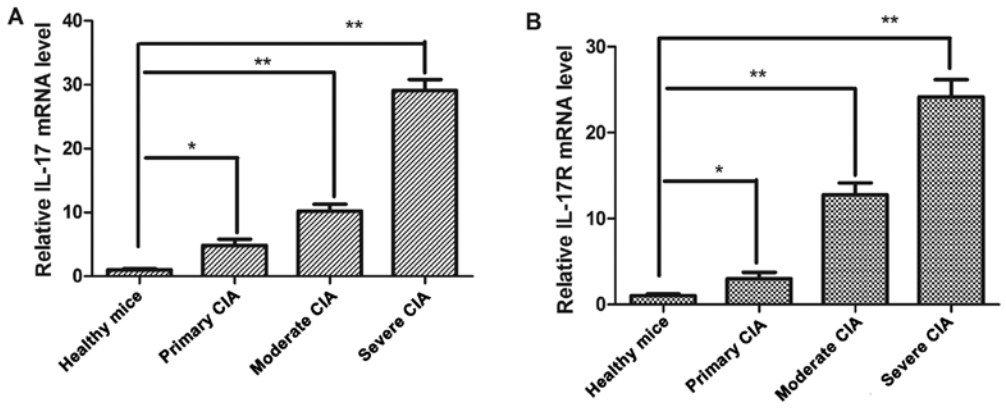

Figure 3. mRNA expression of the pro-inflammatory mediators, interleukin (IL)-17 and IL-17R, detected in peripheral whole blood and in synovial tissue from mice with CIA by real-time RT-PCR. (A) IL-17 in peripheral whole blood of mice with varying degrees of collagen-induced arthritis (CIA) (primary, moderate and severe) and of the healthy controls. (B) IL-17R in synovial tissue of mice with varying degrees of CIA and of healthy controls. Data represent the means ± standard error of the mean (SEM) of triplicate samples. *P<0.05, **P<0.01, from Tukey-Kramer tests.

In vivo inhibition of murine CIA with CMa17Aab and reduction of the humoral immune response against type II collagen. Heterologous type II collagen is widely used as an immunogen for the development of the model of CIA. Antibodies to type II collagen are elevated in mice with CIA. In this study, to assess the therapeutic effects of CMa17Aab on the development of RA, the mouse model of CIA was adopted, in which the disease was induced by the systemic administration of a cocktail of monoclonal antibodies that target various regions of type II collagen, which is one of the major constituents of articular cartilage matrix proteins, followed by lipopolysaccharides (38). The mice were subcutaneously injected with 10 mg/kg CMa17Aab or the same volume of dextrose (DEX) or sodium chloride (NaCl) (untreated group) for nine consecutive days.

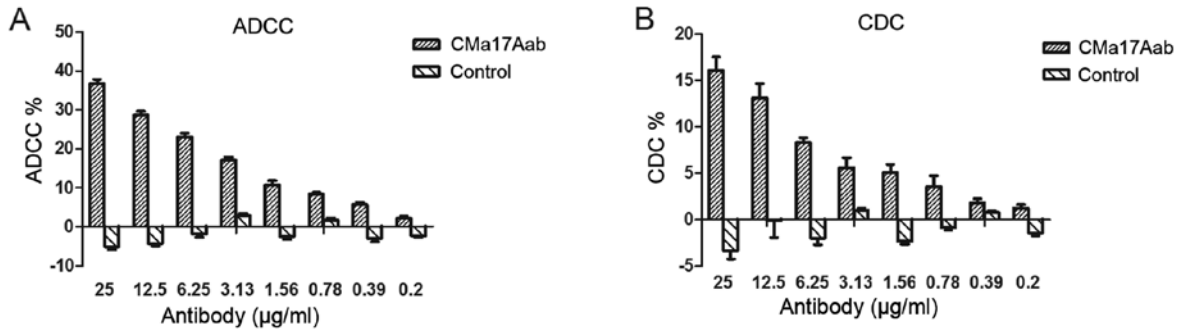

Figure 4. (A) Antibody-dependent cell-mediated cytotoxicity (ADCC) and (B) complement-dependent cytotoxicity (CDC) of CMa17Aab against the free form of interleukin (IL)-17A in IL-17A-expressing cells. Transmembrane IL-17A-expressing HeLa cells were incubated in the presence of various concentrations of CMa17Aab for 1 h. Subsequently, peripheral blood mononuclear cells (PBMCs) for ADCC or human complement-rich serum for CDC were used as effector cells and the source of complement, respectively, and transmembrane IL-17-expressing cells were used as the target cells. The cytotoxicity was calculated by measuring the amount of lactate dehydrogenase (LDH) released from the cytosol into the supernatant. Results are presented as the percentage (%) of cell lysis in the antibody-treated groups compared to 100% lysis in the lysis buffer-treated control group..

As shown in Fig. 5A-C, the CMa17Aab-treated mice showed a significantly reduced progression to severe disease symptoms compared with the saline-treated mice ($P<0.05$, $P<0.01$).

Since the humoral immune response against type II collagen plays a pivotal role in the development of arthritis, we examined the potentially beneficial effects of CMa17Aab on the humoral anti-collagen response, by quantifying the anti-type II collagen IgG level in serum. As shown in Fig. 5D-F, a significant decrease in the serum level of anti-type II collagen IgG was observed in the CMa17Aab-treated mice, compared with the controls ($P<0.01$).

Real-time RT-PCR analysis of RA-related cytokine expression and histopathological analyses of RA synovial grafts in mouse model of CIA. The regulatory effects of CMa17Aab on RA synoviocytes were examined by real-time RT-PCR for eight major genes involved in the pathogenesis of RA: IL-6, IL-8, MMP-3, IL-17, IL-1β, TNF-α, RANKL and IFN-γ. The upregulation of IL-6, MMP-3, IL-8 and IFN-γ by collagen antibody stimulation has been previously demonstrated (39). To evaluate the therapeutic effects of CMa17Aab in mice with varying degrees of CIA, we analyzed the expression of the target genes following independent treatments of mice with varying degrees of CIA with CMa17Aab by RT-PCR (Fig. 5A-C). Our results demonstrated that CIA alone induced an increase in IL-6, IL-8, IFN-α and MMP-3 mRNA expression. Following treatment with CMa17Aab, IFN-α, IL-6, MMP-3 and IL-8 mRNA expression decreased in the mice with primary, moderate and severe CIA compared with the controls (CMa17Aab vs. DEX and NaCl, $P<0.05$ and $P<0.01$, respectively). These results indicate that CMa17Aab downregulates the expression of IL-6, IL-8, MMP-3, IL-17, IL-1β, TNF-α, RANKL and IFN-γ.

In order to observe the changes occurring in RA synovial tissue following treatment with CMa17Aab, X-rays of the ankle joints of the experimental mice were acquired and the ankle joints were then removed and fixed in formalin and decalcified with formic acid. Ten micrometer-thick paraffin-embedded sections were stained with hematoxylin and eosin and the histological changes were observed. As shown in Fig. 6C-E, the untreated mice with CIA showed marked synovial hyperplasia, inflammatory cell influx and destruction of the cartilage and bone. As shown in Fig. 6F-J, there were fewer infiltrating cells in the joints of the CMa17Aab-treated mice compared with the joints from the control mice (treated with DEX) with CIA. This was also accompanied by a decrease in joint synovial proliferation. These changes were statistically significant ($P<0.01$). The pathological symptoms of the CMa17Aab- and DEX-treated CIA mice were attenuated (data not shown).

Discussion

IL-17A is a 17 kDa protein that is secreted predominantly by human memory T cells α and β TCR^+ $CD4^-CD8^-$ thymocytes, mouse peripheral Th17 cells and some innate immune cells (40-42). IL-17A is spontaneously produced in the RA synovium, and is highly expressed in the synovial fluid of pateints and mice with RA. The synergistic action of IL-17 with other pro-inflammatory cytokines, such as IL-1β, TNF-α and IFN-γ provokes local inflammation and amplification of inflammatory responses in RA. IL-17 exerts its effects by binding to IL-17R to stimulate inflammatory cells, which is a central event in mediating cell migration in the pathogenesis of RA (43). In our previous study, we demonstrated that the anti-IL-17 antibody (ScFv) reduced joint inflammation, bone damage and cartilage destruction in RA (44). In addition, treatment with siRNA of the IL-17A receptor I in rat adjuvant arthritis led to a significant suppression of joint inflammation and bone erosion (28). In this study, we constructed a chimeric anti-human IL-17A full-length monoclonal antibody targeting IL-17A, so as to inhibit the pathway generating pathogenic effector TH17 and regulatory T cells (45). In addition, we detected the expression of IL-17 and IL-17RA in the synovium of mice with CIA. Our results demonstrated that IL-17 and IL-17RA were highly expressed in the mice with CIA compared with the healthy mice.

RA is one of the most common human autoimmune diseases, characterized by a chronic inflammatory reaction in the synovium of joints (46). RA is characterized by symmetrical joint swelling. According to the extent of damage to the articular cartilage, RA is divided into primary, moderate and severe RA (47,48). In the primary state of RA, the damage to the articular cartilage is apparent as periarticular soft tissue swelling and joint osteoporosis. In moderate RA, articular cartilage space becomes narrow, indicating extensive cartilage

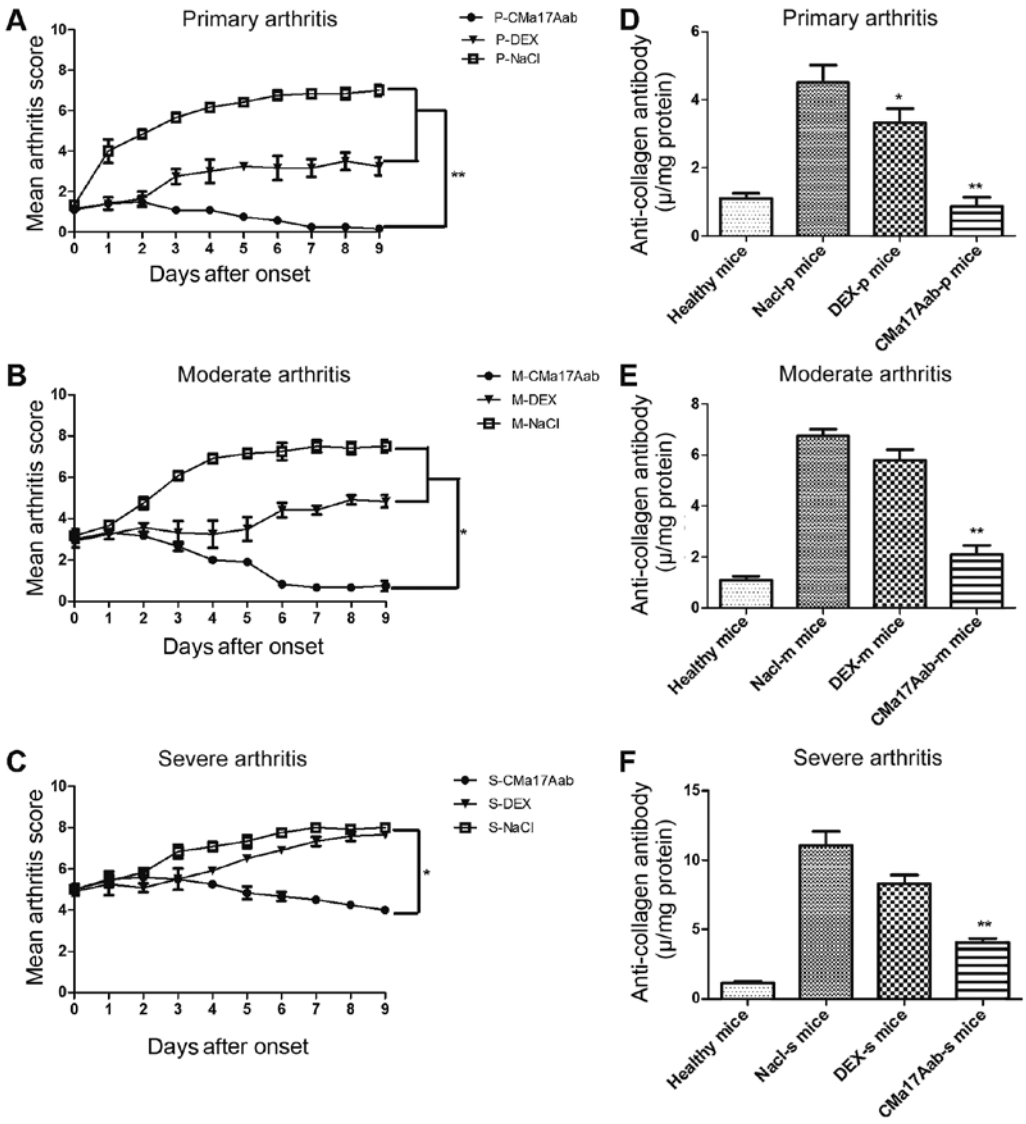

Figure 5. Mean arthritis scores and reduction of anti-collagen type II antibody production in mice with collagen-induced arthritis treated with CMa17Aab, dextrose (DEX) or sodium chloride (NaCl). (A-C) At the time of disease onset (day 0), mice were subcutaneously injected with CMa17Aab (10 mg/kg) or 100 μl DEX or NaCl (non-treated group) for nine consecutive days. The arthritis scores were compared among the non-treated (n=9), the DEX- (n=9) and the CMa17Aab (n=9)-treated groups during the course of the treatment. Scoring results are expressed as the means ± standard error of the mean (SEM). When mice were treated with CMa17Aab, the rate of arthritis development was significantly reduced ($^{*}P<0.05$ and $^{**}P<0.01$). (D-F) Serum was collected on the day of sacrifice and the concentration of anti-collagen IgG was determined by ELISA. Values are expressed as the means and SEM of triplicate samples $^{*}P<0.01$ and $^{**}P<0.01$.

destruction. In severe RA, articular cartilage appears eroded. In this study, based on the clinical arthritis scores of CIA, we examined the therapeutic effects of CMa17Aab in mice with primary, moderate and severe RA. The results confirmed that CMa17Aab is effective in the treatment of RA (compared with the DEX-treated and NaCl-treated mice). However, CMa17Aab was not as effective in the treatment of mice with severe RA compared with mice with primary and moderate RA.

There are a number of known molecular mechanisms that could explain the synergism of IL-17A with other cytokines, such as IL-6, IL-8, MMP-3, IL-17, IL-1β, TNF-α and IFN-γ (49). In this study, we examined the *in vivo* expression of the cytokines, IL-6, MMP-3, IL-8 and IFN-γ, following treatment with CMa17Aab. Our results suggested that these cytokines were highly expressed in the control group (untreated group) and downregulated in the CMa17Aab-treated group. Furthermore, IL-17A plays an important role in human destructive arthritis (50). A previous study demonstrated that in tissue-specific autoimmunity, the IL-17A level is a key determinant of resistance or susceptibility (51). According to our results, the expression of cytokines, such as IL-6, IL-17A, IFN-γ and TNF-α, was upregulated in mice with CIA. However, the expression of these cytokines was relatively lower in the mice with primary RA, compared with the mice with severe RA. As expected, treatment with CMa17Aab resulted in significant decreases in the levels of pro-inflammatory cytokines in the mice with CIA.

Type II collagen is considered the major constituent of articular cartilage in the joings of patients with RA (52). A previous study demonstrated that the immune response to type II collagen may play a role in the damage induced to the articular cartilage of joints (52). The mechanisms underlying

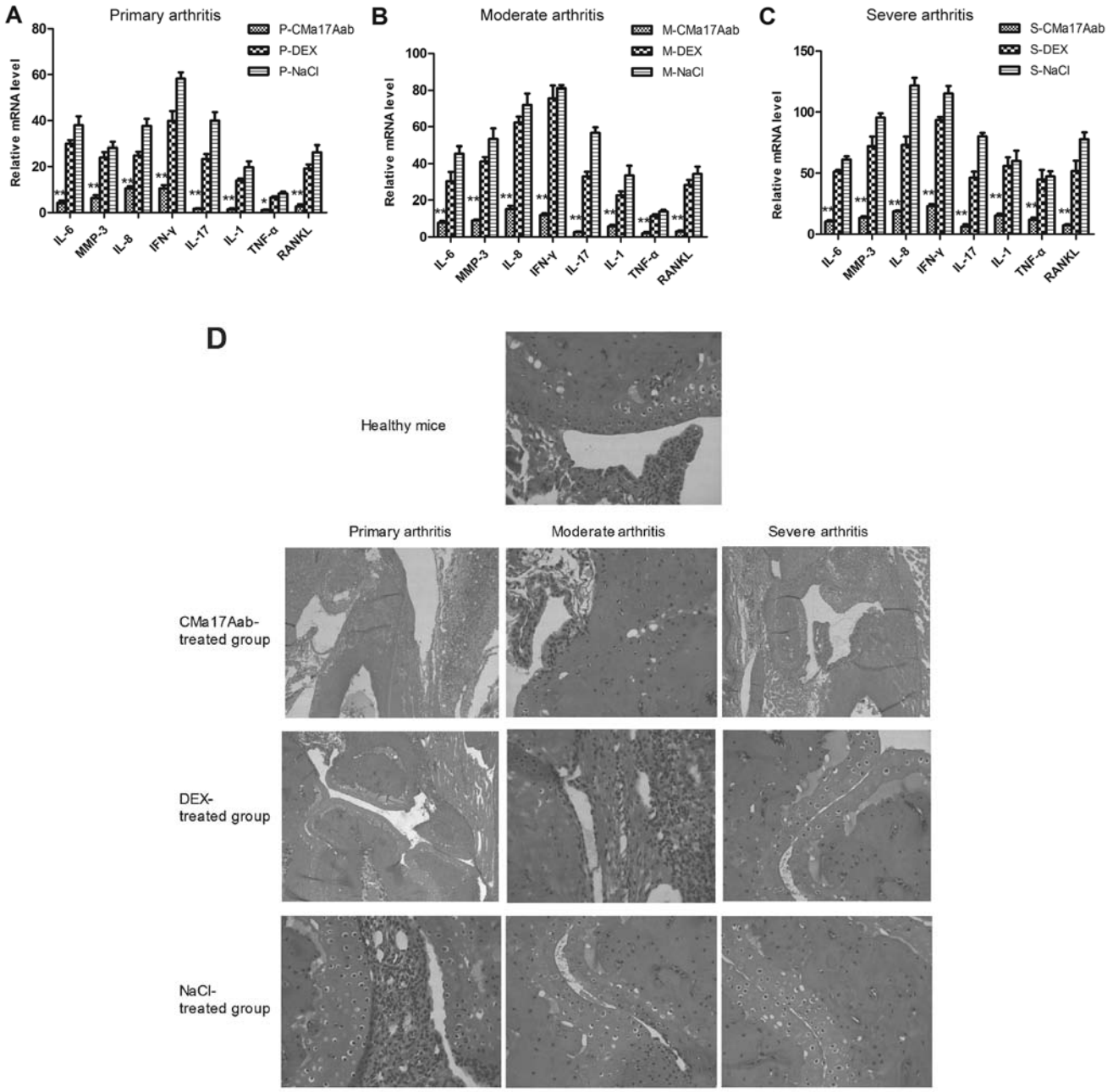

Figure 6. Real-time RT-PCR analysis of rheumatoid arthritis-related cytokines and histopathological analyses of RA synovial grafts from mice with collagen-induced arthritis (CIA). (A-C) mRNA expression of interleukin(IL)-6, IL-8, matrix metalloproteinase (MMP)-3, IL-17, IL-1β, tumor necrosis factor (TNF)-α, receptor activator for nuclear factor-κB ligand (RANKL) and interferon (IFN)-γ in cartilage tissue was quantified by real-time RT-PCR as described in Materials and methods. All the experiments were repeated three times. Data are expressed as the means n-fold difference relative to β-actin ± standard error of the mean (SEM) of triplicate samples. **P<0.01 compared with the DEX-treated and NaCl-treated groups. (D) Synovial tissue samples from healthy mice and mice with rheumatoid arthritis stained with hematoxylin and eosin (H&E). Treatment with CMa17Aab significantly reduced histological damage and bone destruction compared with the controls [dextrose (DEX)- and sodium chloride (NaCl)-treated mice with CIA]. Mice were divided into 9 CIA groups and 1 healthy group (n=9/group). After the mice were sacrificed at the end of the experiment, the hind paws were removed and stained with H&E.

CII-induced RA have been clarified (53,54), with anti-type II collagen antibody secretion from B cells being associated with the development of CIA. Therefore, the serum levels of anti-type II collagen-specific antibodies are markedly high in of patients with RA (55,56). In this study, we examined the effects of CMa17Aab on the abnormal immune response in mice with CIA. The level of serum IgG in the untreated mice with CIA was slightly higher compared with the healthy mice. In addition, following treatment with CMa17Aab, we analyzed the pathological sections of joint issue from the different treatment groups. As expected, the clinical arthritis scores (Fig. 5) indicated that the untreated mice with CIA had marked synovial hyperplasia and inflammatory cell influx, as well as cartilage and bone destruction. In the untreated mice with severe RA, the extent of damage to the articular cartilage was more pronounced.

In conclusion, our study demonstrates that treatment with CMa17Aab exerts beneficial effects by alleviating joint inflammation, cartilage destruction and bone damage in mice with varying degrees of CIA (primary, moderate and severe).

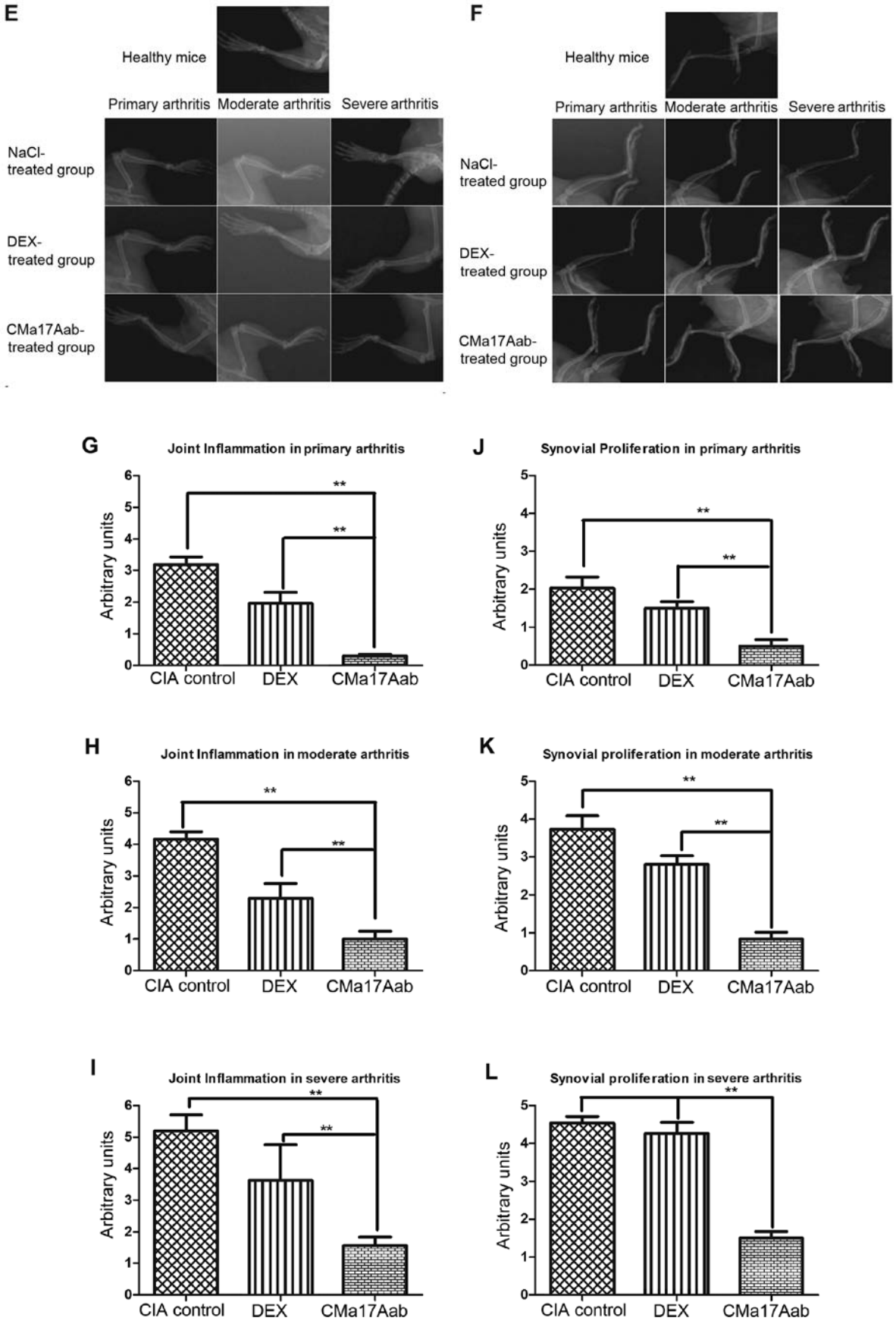

Figure 6. Continued. (E and F) Anteroposterior and lateral image using X-rays to analyze articular cartilage lesions in mice. (H-L) Treatment with CMa17Aab reduced joint degeneration in mice with collagen-induced arthritis (CIA). Joints were harvested from untreated mice with CIA (treated with NaCl; controls), the DEX-treated and CMa17Aab-treated mice with CIA at the end of the experiment (day 40). The joints were fixed in formalin and decalcified with formic acid and then stained with H&E to evaluate inflammation, synovial proliferation and erosion. The presented results are based on the examination of six sections for each treatment group. All values are expressed as the means ± SEM of triplicate samples. $^{**}P<0.01$ CMa17Aab-treated group vs. DEX-treated and NaCl-treated groups (Tukey-Kramer test).

Our study provides evidence that targeting pro-inflammatory cytokines simultaneously can be used as a novel therapeutic approach for patients with RA.

Acknowledgements

We are thankful for the support of the industrialization fund of the Educational Bureau of Heilongjiang Province (1252CGZH29).

References

1. van den Berg WB and Miossec P: IL-17 as a future therapeutic target for rheumatoid arthritis. Nat Rev Rheumatol 5: 549-553, 2009.
2. Onishi RM and Gaffen SL: Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease. Immunology 129: 311-321, 2010.
3. Leipe J, Grunke M, Dechant C, *et al*: Role of Th17 cells in human autoimmune arthritis. Arthritis Rheum 62: 2876-2885, 2010.
4. Maione F, Paschalidis N, Mascolo N, Dufton N, Perretti M and D'Acquisto F: Interleukin 17 sustains rather than induces inflammation. Biochem Pharmacol 77: 878-887, 2009.
5. Chen H, Wang W, Xie H, *et al*: A pathogenic role of IL- 17 at the early stage of corneal allograft rejection. Transpl Immunol 21: 155-161, 2009.
6. Hot A, Zrioual S, Lenief V and Miossec P: IL-17 and tumour necrosis factor α combination induces a HIF-1α-dependent invasive phenotype in synoviocytes. Ann Rheum Dis 71: 1393-1401, 2012.
7. Korn T, Bettelli E, Oukka M and Kuchroo VK: IL-17 and Th17 Cells. Annu Rev Immunol 27: 485-517, 2009.
8. Chabaud M, Durand JM, Buchs N, *et al*: Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 42: 963-970, 1999.
9. Kotake S, Udagawa N, Takahashi N, *et al*: IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 103: 1345-1352, 1999.
10. Chabaud M, Fossiez F, Taupin JL and Miossec P: Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines. J Immunol 161: 409-414, 1998.
11. Jovanovic DV, Di Battista JA, Martel-Pelletier J, *et al*: IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. J Immunol 160: 3513-3521, 1998.
12. Katz Y, Nadiv O and Beer Y: Interleukin-17 enhances tumor necrosis factor alpha-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a 'fine-tuning cytokine' in inflammation processes. Arthritis Rheum 44: 2176-2184, 2001.
13. Kehlen A, Thiele K, Riemann D and Langner J: Expression, modulation and signalling of IL-17 receptor in fibroblast-like synoviocytes of patients with rheumatoid arthritis. Clin Exp Immunol 127: 539-546, 2002.
14. Numasaki M, Lotze MT and Sasaki H: Interleukin-17 augments tumor necrosis factor-alpha-induced elaboration of proangiogenic factors from fibroblasts. Immunol Lett 93: 39-43, 2004.
15. Takahashi H, Numasaki M, Lotze MT and Sasaki H: Interleukin-17 enhances bFGF-, HGF- and VEGF-induced growth of vascular endothelial cells. Immunol Lett 98: 189-193, 2005.
16. Miossec P: Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. Arthritis Rheum 48: 594-601, 2003.
17. Granet C, Maslinski W and Miossec P: Increased AP-1 and NF-kappaB activation and recruitment with the combination of the proinflammatory cytokines IL-1beta, tumor necrosis factor alpha and IL-17 in rheumatoid synoviocytes. Arthritis Res Ther 6: R190-R198, 2004.
18. Chabaud M and Miossec P: The combination of tumor necrosis factor alpha blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an ex vivo model. Arthritis Rheum 44: 1293-1303, 2001.
19. Chabaud M, Page G and Miossec P: Enhancing effect of IL-1, IL-17, and TNF-alpha on macrophage inflammatory protein-3alpha production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines. J Immunol 167: 6015-6020, 2001.
20. Granet C and Miossec P: Combination of the pro-inflammatory cytokines IL-1, TNF-alpha and IL-17 leads to enhanced expression and additional recruitment of AP-1 family members, Egr-1 and NF-kappaB in osteoblast-like cells. Cytokine 26: 169-177, 2004.
21. Chevrel G, Page G, Granet C, Streichenberger N, Varennes A and Miossec P: Interleukin-17 increases the effects of IL-1 beta on muscle cells: arguments for the role of T cells in the pathogenesis of myositis. J Neuroimmunol 137: 125-133, 2003.
22. Kirkham BW, Lassere MN, Edmonds JP, *et al*: Synovial membrane cytokine expression is predictive of joint damage progression in rheumatoid arthritis: a two-year prospective study (the DAMAGE study cohort). Arthritis Rheum 54: 1122-1131, 2006.
23. Yao Z, Spriggs MK, Derry JM, *et al*: Molecular characterization of the human interleukin (IL)-17 receptor. Cytokine 9: 794-800, 1997.
24. Tachihara A, Jin E, Matsuoka T, *et al*: Critical roles of capillary endothelial cells for alveolar remodeling in nonspecific and usual interstitial pneumonias. J Nippon Med Sch 73: 203-213, 2006.
25. Tian E, Sawyer JR, Largaespada DA, Jenkins NA, Copeland NG and Shaughnessy JD Jr: Evi27 encodes a novel membrane protein with homology to the IL17 receptor. Oncogene 19: 2098-2109, 2000.
26. Shi Y, Ullrich SJ, Zhang J, *et al*: A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity. J Biol Chem 275: 19167-19176, 2000.
27. Lee J, Ho WH, Maruoka M, *et al*: IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. J Biol Chem 276: 1660-1664, 2001.
28. Zrioual S, Toh ML, Tournadre A, *et al*: IL-17RA and IL-17RC receptors are essential for IL-17A-induced ELR$^+$ CXC chemokine expression in synoviocytes and are overexpressed in rheumatoid blood. J Immunol 180: 655-663, 2008.
29. Miljkovic D, Cvetkovic I, Vuckovic O, Stosic-Grujicic S, Mostarica Stojkovic M and Trajkovic V: The role of interleukin-17 in inducible nitric oxide synthase-mediated nitric oxide production in endothelial cells. Cell Mol Life Sci 60: 518-525, 2003.
30. Lubberts E, Koenders MI, Oppers-Walgreen B, *et al*: Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion. Arthritis Rheum 50: 650-659, 2004.
31. Genovese MC, Van den Bosch F, Roberson SA, *et al*: LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. Arthritis Rheum 62: 929-939, 2010.
32. Nardelli DT, Burchill MA, England DM, Torrealba J, Callister SM and Schell RF: Association of CD4$^+$ CD25$^+$ T cells with prevention of severe destructive arthritis in *Borrelia burgdorferi*-vaccinated and challenged gamma interferon-deficient mice treated with anti-interleukin-17 antibody. Clin Diagn Lab Immunol 11: 1075-1084, 2004.
33. Nakajima H, Takamori H, Hiyama Y and Tsukada W: The effect of treatment with interferon-gamma on type II collagen-induced arthritis. Clin Exp Immunol 81: 441-445, 1990.
34. Stuart JM, Townes AS and Kang AH: Type II collagen-induced arthritis. Ann NY Acad Sci 460: 355-362, 1985.
35. Toy D, Kugler D, Wolfson M, *et al*: Cutting edge: interleukin 17 signals through a heteromeric receptor complex. J Immunol 177: 36-39, 2006.
36. Fan Y, Weifeng W, Yuluan Y, Qing K, Yu P and Yanlan H: Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of coxsackievirus b3-induced viral myocarditis reduces myocardium inflammation. Virol J 8: 17, 2011.
37. Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H and Maslinski W: High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J Immunol 164: 2832-2838, 2000.
38. Bas DB, Su J, Sandor K, *et al*: Collagen antibody-induced arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency. Arthritis Rheum 64: 3886-3896, 2012.
39. Nandakumar KS and Holmdahl R: Collagen antibody induced arthritis. Methods Mol Med 136: 215-223, 2007.
40. Yao Z, Painter SL, Fanslow WC, *et al*: Human IL-17: a novel cytokine derived from T cells. J Immunol 155: 5483-5486, 1995.
41. Miyamoto M, Prause O, Sjostrand M, Laan M, Lötvall J and Lindén A: Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways. J Immunol 170: 4665-4672, 2003.

42. Kennedy J, Rossi DL, Zurawski SM, *et al*: Mouse IL-17: a cytokine preferentially expressed by alpha beta TCR + CD4-CD8-T cells. J Interferon Cytokine Res 16: 611-617, 1996.
43. Zhang R, Qian J, Guo J, Yuan YF and Xue K: Suppression of experimental autoimmune uveoretinitis by Anti-IL-17 antibody. Curr Eye Res 34: 297-303, 2009.
44. Zhang Y, Ren G, Guo M, *et al*: Synergistic effects of interleukin-1beta and interleukin-17A antibodies on collagen-induced arthritis mouse model. Int Immunopharmacol 15: 199-205, 2013.
45. Miossec P: IL-17 and Th17 cells in human inflammatory diseases. Microbes Infect 11: 625-630, 2009.
46. Bazzani C, Filippini M, Caporali R, *et al*: Anti-TNFalpha therapy in a cohort of rheumatoid arthritis patients: clinical outcomes. Autoimmun Rev 8: 260-265, 2009.
47. Gandjbakhch F, Conaghan PG, Ejbjerg B, *et al*: Synovitis and osteitis are very frequent in rheumatoid arthritis clinical remission: results from an MRI study of 294 patients in clinical remission or low disease activity state. J Rheumatol 38: 2039-2044, 2011.
48. Emery P, McInnes IB, van Vollenhoven R and Kraan MC: Clinical identification and treatment of a rapidly progressing disease state in patients with rheumatoid arthritis. Rheumatology (Oxford) 47: 392-398, 2008.
48. Hornung N, Ellingsen T, Attermann J, Stengaard-Pedersen K and Poulsen JH: Patients with rheumatoid arthritis treated with methotrexate (MTX): concentrations of steady-state erythrocyte MTX correlate to plasma concentrations and clinical efficacy. J Rheumatol 35: 1709-1715, 2008.
50. Liu Y, Mei J, Gonzales L, *et al*: IL-17A and TNF-alpha exert synergistic effects on expression of CXCL5 by alveolar type II cells in vivo and in vitro. J Immunol 186: 3197-3205, 2011.
51. Leung S, Liu X, Fang L, Chen X, Guo T and Zhang J: The cytokine milieu in the interplay of pathogenic Th1/Th17 cells and regulatory T cells in autoimmune disease. Cell Mol Immunol 7: 182-189, 2010.
52. Myers LK, Rosloniec EF, Cremer MA and Kang AH: Collagen-induced arthritis, an animal model of autoimmunity. Life Sci 61: 1861-1878, 1997.
53. Griffiths MM, Nabozny GH, Hanson J, *et al*: Collagen-induced arthritis and TCRs in SWR and B10.Q mice expressing an Ek alpha transgene. J Immunol 153: 2758-2768, 1994.
54. Durie FH, Fava RA and Noelle RJ: Collagen-induced arthritis as a model of rheumatoid arthritis. Clin Immunol Immunopathol 73: 11-18, 1994.
55. Nabozny GH, Bull MJ, Hanson J, Griffiths MM, Luthra HS and David CS: Collagen-induced arthritis in T cell receptor V beta congenic B10.Q mice. J Exp Med 180: 517-524, 1994.
56. Kadowaki KM, Matsuno H, Tsuji H and Tunru I: $CD4^+$ T cells from collagen-induced arthritic mice are essential to transfer arthritis into severe combined immunodeficient mice. Clin Exp Immunol 97: 212-218, 1994.