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Original article

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Abstract

Objective We earlier found that TNFα but not interleukin (IL)-17 is indispensable in the pathogenesis of spontaneously occurring rheumatoid arthritis (RA)-like disease in our newly established FcγRIIB-deficient C57BL/6 (B6) mouse model, designated KO1. Here, we examined the role of IL-6 in the pathogenesis of RA features in KO1, with particular reference to cartilage and bone destruction in arthritic joints.

Methods To evaluate the preventive effect of MR16-1, a rat anti-mouse IL-6 receptor (IL-6R) mAb, 4-month-old preclinical KO1 mice were divided into three groups: the first treated with MR16-1 for 6 months, the second treated with normal rat IgG, as a control, and the third left untreated. The incidence and severity of arthritis, immunological abnormalities, and transcription levels of receptor activator of NF-κB ligand (RANKL), osteoprotegerin (OPG), and inflammatory cytokines/chemokines in ankle joint tissues were compared among the three groups. The therapeutic effect of MR16-1 was examined by treating 7-months-old KO1 mice in the early stages of arthritis.

Results Compared with the findings in the KO1 mice left untreated or treated with normal rat IgG, the development of arthritis was markedly suppressed in mice with MR16-1treatment started from preclinical stages. The suppression was associated with the decrease in production of autoantibodies, rheumatoid factors (RF) and anti-cyclic citrullinated peptide (CCP). Histologically, marked synovitis, pannus formation, and cartilage and bone destruction associated with the increase in tartrate-resistant acid phosphatase (TRAP)-positive osteoclast generation were evident in the two control groups; however, these findings were virtually absent in MR16-1-treated mice. Real-time PCR analysis revealed that the up-regulated expression levels of MCP-1, IL-6, and TNFα, and the aberrantly high RANKL/OPG expression ratio in synovial joint tissues from the two control groups of mice with overt arthritis were significantly suppressed in MR16-1-treated mice. In mice with therapeutic MR16-1 treatment, there was no progression in arthritis score and the RANKL/OPG ratio in joint tissues was significantly suppressed.

Conclusions Administration of an anti-IL-6R mAb ameliorated spontaneously occurring RA-like disease features, indicating that IL-6, as well as TNFα, plays a pivotal role in the pathogenesis of RA in KO1 mice. Current studies showed that, in addition to the role in enhancing autoantibody production, IL-6 promotes synovial tissue inflammation and osteoclastogenesis, leading to the severe synovitis with pannus formation and the progressive cartilage and bone destruction in multiple joints.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease, characterized by the development of synovial tissue inflammation in multiple joints followed by synovial hyperplasia with pannus formation and the progressive destruction of cartilage and bone mediated by enhanced proliferation of activated osteoclasts. The process of osteoclastogenesis is controlled by the interaction of receptor activator of NF-κB (RANK) expressed on osteoclast precursors with its ligand RANKL expressed on synovial fibroblasts, osteoblasts, and Th17 cells [**1,2**]. RANKL-mediated osteoclastogenesis is counterbalanced by the physiologically expressed decoy receptor, osteoprotegerin (OPG) [**3**]. Accumulating evidence has indicated that high expression levels of proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1, IL-6, and IL-17 in inflamed synovial tissues may play a pivotal role in the processes of both synovial hyperplasia and osteoclastogesis [**4**].

To evaluate the therapeutic potentials of targeting the proinflammatory cytokines for RA, extensive clinical studies of RA patients have been carried out by the administration of either inhibitory antibodies or antagonistic proteins. Clinical trials done by blocking the TNFα signal using anti-TNFα antibodies or TNF receptor-IgG Fc

fusion protein as well as by blocking IL-1 signal using recombinant IL-1 receptor antagonist (IL-1Ra) showed that, while all these therapeutic approaches have been on the whole successful, the effects of treatments were variable and not always satisfactory for all patients [**5-7**]. The same was true in phase II clinical studies of RA patients under anti-IL-17 antibody administration [**8**].

Using several kinds of murine RA model, such cytokine blocking approaches have been carried out; however, the therapeutic effects varied considerably, depending on the RA mouse models examined. For instance, although the anti-TNFα antibody therapy was effective in the type II collagen-induced arthritis (CIA) of DBA mice [**9,10**], severe CIA still developed in TNFα-deficient C57BL/6 (B6) mice [**11**]. In the K/BxN serum transfer arthritis model, there was no evidence of any particular requirement of TNFα for bone destruction [**12**]. As for the role of IL-17, genetic IL-17 deficiency was reported to suppress completely the arthritis spontaneously occurring in ZAP-70-mutant SKG mice [**13**] and in IL-1Ra-deficient mice [**14**]. However, the lack of IL-17 was not fully sufficient to prevent CIA [**15**], and had no effect on the human cartilage proteoglycan-induced arthritis [**16**]. These observations, taken collectively, clearly indicate that different mechanisms underlie the pathogenesis of RA in individual

patients as well as in individual mouse models; thus, further studies using several kinds of arthritis model are valuable to clarify the therapeutic mechanisms of each cytokine inhibitor for RA.

Recently, as the second-generation anti-cytokine approach, the therapeutic effects of IL-6 signal blockade using anti-IL-6 receptor (IL-6R) antibodies in RA patients were examined. A series of clinical trials have demonstrated an excellent efficacy of tocilizumab, a humanized anti-human IL-6R mAb, in trial designs either with or without methotrexate, an anchor drug for RA therapy [**17-19**], showing that anti-IL-6R antibodies would be an additional promising therapeutic option for RA patients. Studies in CIA mouse model likewise revealed that the treatment with anti-mouse IL-6R mAb, MR16-1, was effective to ameliorate arthritis [**20**]. However, both the K/BxN serum transfer arthritis and the anti-type II collagen mAb-induced arthritis normally developed in mice with genetic deficiency of IL-6 [**12,21**]. Thus, studies using additional arthritis mouse models are helpful to clarify the mechanism of the effect of IL-6 signal blockade on arthritis.

We recently found that a B6 mouse strain genetically deficient in inhibitory IgG Fc receptor IIB (FcγRIIB), designated KO1, spontaneously develops human RA-like

disease features with marked synovial hyperplasia and severe cartilage and bone destruction in multiple joints [**22**]. To evaluate the role of TNFα and IL-17 in the pathogenesis of this arthritis, we earlier introduced genetic deficiency of TNFα and IL-17 in the KO1 mouse strain [**23**]. The results showed that TNFα−deficient KO1 mice did not develop arthritis at all; however, IL-17-deficient KO1 mice developed severe arthritis with comparable incidence and severity as those found in KO1 mice [**23**].

In the present studies, we treated KO1 mice with MR16-1 in order to clarify the role of IL-6 in the pathogenesis of RA-like disease in KO1 mice. Our studies revealed that the MR16-1 treatment suppressed not only RA-related autoantibody production but also RANKL-mediated osteoclastogenesis through normalizing the RANKL/OPG ratio, indicating that IL-6, as well as TNFα, plays a pivotal role in the pathogenesis of RA in this model mouse strain.

Materials and methods

Mice

Arthritis-prone KO1 is an FcγRIIB-deficient B6 congenic line [**22**], obtained by backcrossing the originally constructed FcγRIIB-deficient mice on a hybrid (129 x B6)

background [**24**] into a B6 background for over 12 generations. Only female mice were analyzed in the present study. All mice were housed under identical specific-pathogen-free conditions, and all experiments were performed in accordance with our institutional guidelines.

Scoring of arthritis

Ankle joint swelling was examined by inspection and arbitrarily scored as follows: 0, no swelling; 1, mild swelling; 2, moderate swelling; and 3, severe swelling. Scores for both ankle joints were summed for each mouse, and mice with a score of 2 or over were considered positive for arthritis.

***In vivo* administration of anti-IL-6R mAb**

To examine the preventive effect of neutralizing mAb against IL-6R on the development of RA, 4-month-old preclinical KO1 mice were randomly divided into three groups. Each group of 15 mice was left untreated, treated with normal rat IgG (Sigma Chemical Co.), or treated with rat anti-mouse IL-6R mAb (MR16-1, rat IgG1, a kind gift from Chugai Pharmaceutical, Tokyo, Japan) [**25**]. Two hundred micrograms of rat IgG or MR16-1 was administered i.p. twice a week for 6 months. To evaluate the therapeutic effect of MR16-1 treatment, 7-month-old mice in the early stages of arthritis with a

score 1 or 2 were selected and injected i.p. with 200 micrograms of MR16-1 twice a week for 2 months.

Histopathology

Joint tissues were decalcified in 10% EDTA in 0.1 M Tris buffer (pH 7.4), fixed in 4% paraformaldehyde, and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin, and also stained for tartrate-resistant acid phosphatase (TRAP) using the TRAP/ALP stain kit (Wako Pure Chemical Industries Ltd.).

Serum levels of antibodies

Serum levels of IgG class anti-cyclic citrullinated peptide (CCP) antibodies were measured employing commercially available kits (Cosmic Corporation) using anti-mouse IgG second antibodies and are expressed as relative units according to the manufacturer's instructions. Serum levels of rheumatoid factor (RF) were measured using an ELISA, as previously described [**26**]. Briefly, an ELISA plate pre-coated with mouse IgG Fc fragment (OEM Concepts) was incubated with appropriately diluted mouse serum samples, washed, and then incubated with peroxidase-conjugated rat anti-mouse κ chain antibodies (BD Biosciences Pharmingen). Activities for RF are expressed in units referring to a standard curve obtained by serial dilution of a standard

serum pool from 4-6-month-old MRL/*lpr* mice containing 1000 unit activities/ml. Serum levels of IgG class anti-double-stranded (ds) DNA were measured using an ELISA plate pre-coated with 5 μg/ml calf thymus dsDNA (Sigma-Aldrich). DNA-binding activities are expressed in units, as previously described [**22**].

Flow cytometric analysis

Spleen cells were four-color-stained with the following mAbs: anti-B220, anti-CD69, anti-CD138, anti-CD3, anti-CD4, anti-CD8, and anti-CD25, and with peanut agglutinin (PNA). Foxp3 staining was performed using Foxp3/transcription Factor Staining buffer set (eBioscience). For peripheral monocyte staining, peripheral leukocytes were stained with anti-CD11b mAb. Stained cells were analyzed using a FACSAria cytometer and FlowJo software (Tree Star, Inc., Ashland, OR), excluding dead cells in forward and side scatter cytogram.

For intracellular cytokine staining, spleen cells were stimulated with PMA(0.2 μg/ml)/ionomycin(2 μg/ml) in the presence of Golgi-Stop (BD Bioscience) for 5 h and stained with 7-ADD (eBioscience) (staining for dead cells) and with Pacific Blue-labeled anti-CD4 mAb. Stained cells were then fixed and permeabilized using BD Cytofix/Cytoperm (BD Bioscience), followed by staining with FITC-labeled anti-TNFα and anti-IFNγ mAbs, and PE-labeled anti-IL-4 and anti-IL-7 mAbs. Stained cells were analyzed as above, excluding 7-ADD-positive cells.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from ankle joint tissues using QIAGEN RNeasy Lipid Tissue Minikit (Cat. No. 74804). Briefly, ~25 mg of ankle joint tissue was added in 500 μl of QIAzol lysis reagent in a 2 ml tube containing 5-mm-diameter zirconia beads (Hirasawa YTZ-5) and homogenized on TissueLyser (Qiagen) for 1 min at 30 Hz. Total RNA was extracted from homogenized materials using Minikit according to the manufacturer's instructions, and the single-stranded cDNA was synthesized using an oligo(dT)-primer with Superscript II First-Strand Synthesis kit (Invitrogen). The cDNA product was used for qRT-PCR. The data were normalized to β-actin as a reference. The primer pairs used were as follows: RANKL (forward) 5'-TGTACTTTCGAGCGCAGATG-3', (reverse) 5'-AGGCTTGTTTCATCCTCCTG-3'; OPG (forward) 5'-TGAGTGTGAGGAAGGGCGTTA-3', (reverse) 5'-CCATCTGGACATTTTTTGCAAA-3'; MCP-1 (forward) 5'-AGGTCCCTGTCATGCTTCTG-3', (reverse) 5'-TCTGGACCCATTCCTTCTTG-3'; RANTES (forward) 5'-TCGTGCCCACGTCAAGGAGTATTT-3', (reverse) 5'-TCTTCTCTGGGTTGGCACACACTT-3'; CX3CL1 (forward) 5'-CGCGTTCTTCCATTTGTGTA-3', (reverse) 5'- CTGTGTCGTCTCCAGGACAA-3';

IL-6 (forward) 5’-GAGGATACCACTCCCAACAGACC-3’, (reverse) 5’-AAGTGCATCATCGTTGTTCATACA-3’; TNFα (forward) 5’-TATGGCCCAGACCCTCAC-3’, (reverse) 5’-GGTTGTCTTTGAGATCCATGC-3’; IFNγ (forward) 5'-AAGACAATCAGGCCATCAGC-3’, (reverse) 5’-ATCAGCAGCGACTCCTTTTC-3’; IL-17 (forward) 5’-TCTCTGATGCTGTTGCTGCT-3’, (reverse) 5’-GACCAGGATCTCTTGCTGGA-3’; Foxp3 (forward) 5’-TCCTTCCCAGAGTTCTTCCA-3’, (reverse) 5’-AGGGATTGGAGCACTTGTTG-3’; RORγt (forward) 5’-GCCCTGTGTTTTTCTGAGGA-3’, (reverse) 5’-AGGGGATTCAACATCAGTGC-3’; and β-actin (forward) 5’-TGGGTATGGAATCCTGTGG-3’, (reverse) 5’-GTACTTGCGCTCAGGAGGAG-3’. The quantity was normalized using the $2^{-\Delta\Delta CT}$ method. Values of B6 mice were designated as 1, and values of each of the three groups of mice were evaluated as fold change compared with the values in B6 mice.

Statistics

Statistical analysis was carried out using the Kaplan-Meier method for the incidence of

arthritis, Mann-Whitney's *U* test for antibody levels and qRT-PCR analysis, and Student's *t*-test for arthritis score and flow cytometric analysis. A value of $P < 0.05$ was considered as statistically significant.

Results

Preventive effect of MR16-1 treatment on incidence and severity of arthritis

Four-month-old disease-free KO1 mice were divided into three groups, namely untreated, normal rat IgG-treated, and MR16-1-treated groups. Fig. 1a compares the cumulative incidence and severity of arthritis in ankle joints among these three groups of mice. Untreated KO1 mice spontaneously developed arthritis with swelling and limited mobility of the ankle joints symmetrically after 5 months of age, and the severity of arthritis increased with age. KO1 mice treated with normal rat IgG developed severe arthritis with comparable incidence and severity to that observed in the untreated KO1 mice. In contrast, the arthritis was markedly suppressed in MR16-1-treated KO1 mice and its incidence was only 13%, even by 10 months of age. Fig. 1b shows representative macroscopic findings of hind paws of the three groups of mice at 10 months of age. Histological examination revealed severe synovitis with

remarkable pannus formation and destruction of cartilage and bone in untreated KO1 and normal rat IgG-treated KO1 mice (Fig. 1c upper panel). TRAP staining showed an increase in the number of TRAP-positive osteoclasts at the resorption lacuna on the surface of cartilage and bone in untreated KO1 and normal rat IgG-treated KO1 mice (Fig. 1c lower panel). These changes were virtually absent in MR16-1-treated KO1 mice.

Effect on serum autoantibody levels, activation status of lymphocytes, and peripheral monocyte frequencies

Fig. 2 compares the serum levels of RF and IgG class autoantibodies against CCP and dsDNA among untreated, normal rat IgG-treated, and MR16-1-treated groups of mice at 8 months of age. Levels of RF and anti-CCP antibodies were significantly suppressed in MR16-1-treated KO1 mice compared with those in untreated and normal rat IgG-treated KO1 mice. Levels of anti-dsDNA antibodies in MR16-1-treated KO1 mice were also significantly suppressed compared with those in normal rat IgG-treated KO1 mice.

We then examined the spleen weight and the frequencies of splenic lymphocyte subsets and peripheral monocytes in mice at 9~10 months of age. As shown in Table 1, while there was no difference in the spleen weight, the frequencies of total B cells,

PNA^+ germinal center B cells, $CD138^+$ plasma cells, $CD3^+$ T cells, and $CD8^+$ T cells among the three groups of mice, the frequencies of $CD69^+$ activated B cells per total B cells and $CD4^+$ T cells per total cells were significantly decreased in MR16-1-treated KO1 mice compared with those in untreated and normal rat IgG-treated KO1 mice. Among $CD4^+$ T cells, the frequency of $CD25^+Foxp3^+$ T regulatory (Treg) cells did not differ significantly among the three groups of mice. We also examined the frequencies of peripheral monocytes, which contain osteoclast precursors [**27**]. Compared with normal B6 mice with monocyte frequencies under 10% [**28**], the markedly high frequencies of monocytes over 50% was observed in all the three groups of mice. There were no significant differences among the three groups of mice.

We further examined the frequencies of $CD4^+$ T cell subsets by the intracellular cytokine staining of *in vitro* PMA/ionomycin-stimulated spleen cells. As shown in Table 2, *in vitro* stimulated $CD4^+$ T cells mainly produced TNFα and IFNγ, while both IL-4 and IL-17 were produced by a limited population. When the frequencies of these cytokine-producing $CD4^+$ T cells were compared between untreated and MR16-1-treated groups of mice, the frequency of $TNF\alpha^+$ T cells was significantly decreased in MR16-1-treated KO1 mice, while there was no difference in the

frequencies of IFNγ$^+$ T cells, IL-4$^+$ T cells, and IL-17$^+$ T cells per CD4$^+$ T cells between the two groups of mice.

qRT-PCR analysis of RANKL/OPG and cytokine/chemokine mRNA expression levels in ankle joints

To evaluate the transcription levels of RANKL, OPG, cytokines, and chemokines in ankle joint tissues, qRT-PCR analysis was performed using mRNA extracted from joint tissues of each of four 8~9-month-old mice from the three groups. Data of normal B6 mice were used as a relative control. The expression levels in the three groups of mice were evaluated as fold change compared with the level in B6 mice tentatively designated as 1. As shown in Fig. 3a, the expression level of RANKL in MR16-1-treated KO1 mice was significantly lower than the levels in untreated and normal rat IgG-treated KO1 mice. As for the OPG expression levels, all the three groups showed the levels lower than the level in B6 mice. Nevertheless, when the levels were compared among the three groups, the level in MR16-1-treated KO1 mice was significantly higher than the levels in untreated and normal rat IgG-treated KO1 mice. Accordingly, when calculating the RANKL/OPG ratio (Fig. 3b), the average ratio in MR16-1-treated KO1 mice was almost the same as that in normal B6 mice. This was in

contrast to the findings in untreated and normal rat IgG-treated KO1 mice, in which these ratios were over 10 times higher than the ratio in normal B6 mice.

As MCP-1, RANTES, and CX3CL1 have been shown to be important chemokines involved in the pathogenesis of RA [**29**], we compared their expression levels in ankle joint tissues. As shown in Fig. 3c, the MCP-1 expression levels were markedly up-regulated in untreated and normal rat IgG-treated KO1 mice with overt arthritis, while arthritis-free MR16-1-treated KO1 mice showed significantly lower levels. As for the RANTES expression levels, the three groups of mice showed low levels and there was no difference among them. It was noted that CX3CL1 expression levels in the three groups of mice were lower than the level in control B6 mice, and that the levels in untreated and normal rat IgG-treated KO1 mice with overt arthritis were significantly lower than that in arthritis-free MR16-1-treated KO1 mice. Thus, it appears that MCP-1 plays a pivotal role in the proscess of inflammation in arthritic joints of KO1 mice.

Fig. 3d compares the expression levels of IL-6, TNFα, and IFNγ among the three groups of mice. The levels of IL-6 were markedly up-regulated in untreated and normal rat IgG-treated KO1 mice compared with the level in MR16-1-treated KO1 mice. Intriguingly, the IL-6 expression level in MR16-1-treated KO1 mice was to a great

degree suppressed, even to below the level in normal B6 mice, suggesting that IL-6 secreting cells in ankle joints is under the control of autocrine mechanism [**30**]. The levels of TNFα were also markedly up-regulated in the former two arthritis-prone groups of mice, but the level in MR16-1-treated KO1 mice was the same as the level in normal B6 mice. IFNγ expression levels in the three groups were comparable to the level in B6 mice, and there was no difference in expression levels among the three groups. IL-17 expression was undetectable in ankle joint tissues from B6 and the three groups of mice in our experiment, as previously described [**23**].

Since IL-6 was shown to play an important role in the regulation of Th17/Treg cell balance [**31**], we examined the effect of IL-6 signal blockade on the expression levels of Foxp3 and RORγt, the transcription factors specific for the development of Treg cells and Th17 cells, respectively. The results showed that there was no *in vivo* effect of MR16-1 treatment on their expression levels in ankle joint tissues in our arthritis model (Fig. 3e).

Therapeutic effect of MR16-1 treatment

To further examine the therapeutic effect of MR16-1 treatment, 7-month-old KO1 mice with arthritis of the score 1 or 2 were selected and divided into two groups. One group

was treated with MR16-1 for 2 months and the other group was left untreated. The arthritis score in mice left untreated was increased with age; however, there was no such increase in the score in MR16-1-treated group of mice (Fig. 4a).

We also evaluated the transcription levels of RANKL, OPG, MCP-1, IL-6, and TNFα in ankle joint tissues using qRT-PCR analysis. As in the case of preventive treatment, RANKL expression level was decreased and OPG expression level was increased in therapeutic MR16-1 treatment (Fig. 4b), resulting in the significant decrease in RANKL/OPG ratio (Fig. 4c), compared with the findings in untreated mice. In contrast, the therapeutic MR16-1 treatment showed no effect on the expression levels of MCP-1 (Fig. 4d) and TNFα (Fig. 4e) in ankle joint tissues, indicating that IL-6 signal blockade may be ineffective to suppress the ongoing inflammatory cytokine/chemokine production. The IL-6 expression level was lower in MR16-1-treated mice than found in untreated mice; however, the level was much higher when compared with that in normal B6 mice (Fig. 4e), the finding quite different from that in the case of preventive treatment (Fig. 3d).

Discussion

The severity of arthritis spontaneously occurring in KO1 mice was markedly suppressed with the treatment of an inhibitory mAb against IL-6R, MR16-1. The marked synovitis with inflammatory cell infiltration, pannus formation, TRAP-positive osteoclast generation, and the destruction of cartilages and bones observed in untreated and normal rat IgG-treated KO1 mice were seldom observed in MR16-1-treated KO1 mice. This suppression was associated with the decrease in production of autoantibodies RF and anti-CCP. These findings clearly demonstrate that, in addition to the role in enhancing the autoantibody production, IL-6 plays a pivotal role in the inflammatory response and osteoclastogenesis in arthritic tissues in KO1 mice. It was notable that, when the average ratio of RANKL to OPG expression levels in joint tissues was examined, while the ratios in arthritis-prone untreated and normal rat IgG-treated KO1 mice were both over 10 times higher than the ratio in normal B6 mice, such aberrant ratios were ameliorated in KO1 mice with both preventive and therapeutic treatments of MR16-1. As OPG is a decoy receptor for RANKL and suppresses RANKL-mediated osteoclastogenesis [**3**], normalization of the aberrantly high RANKL/OPG ratio appears to be responsible for the protective effect of MR16-1 on the joint destruction.

RA is an autoantibody-mediated autoimmune disease, in which IgG immune complexes (ICs) may trigger the joint inflammation and induce a marked cellular infiltrate including macrophages, mast cells, $CT4^+$ T cells, $CD8^+$ T cells, and B cells. The exact mechanisms for the inflammatory cell infiltration are unidentified; however, it appears that inflammatory cytokines/chemokines play a pivotal role for the disease progression [**4**]. Our arthritis-prone KO1 mice lack the expression of inhibitory FcγRIIB, which is usually expressed on B cells and myeloid cells such as macrophages and mast cells. The lack of FcγRIIB expression on B cells induces the breakdown of self-tolerance and the production of IgG autoantibodies [**32**]. The resultant IgG ICs formed in the joint tissues stimulate macrophages via activated IgG Fc receptors to produce several kinds of inflammatory cytokines, such as TNFα and IL-6 [**4**]. This step may be augmented in KO1 mice, since KO1 macrophages lack inhibitory FcγRIIB. Thus, it is feasible to speculate that activated macrophages play a pivotal role in the pathogenesis of arthritis in KO1 mice. This is consisted with the present study as well as our previous study [**23**], showing that both IL-6 and TNFα are indispensable in arthritis of KO1 mice.

IL-6 is a multifunctional cytokine. It was originally identified as a B-cell

differentiation factor that induces the terminal maturation of B cells into antibody-producing plasma cells. However, later studies have shown that IL-6 acts to activate and differentiate not only B cells but also T cells, hepatocytes, and hematopoietic progenitor cells [**33**]. In the present study, the MR16-1 treatment resulted in down-regulation of autoantibody production associated with the decrease in the frequency of $CD69^+$ activated B cells as well as in the frequency of $CD4^+$ T cells in the spleen. Intracellular cytokine staining analysis revealed that the frequency of TNFα-producing $CD4^+$ T cells was decreased in MR16-1-treated mice. Although IL-6 was shown to regulate Th17/Treg cell balance [**31**], our qRT-PCR and intracellular cytokine staining analysis in our model revealed that there was no detectable *in vivo* effect of IL-6 signal blockade on this balance. It has been reported that osteoclast precursors arise from the monocyte/macrophage lineage of cells derived from hematopoietic progenitor cells [**27**] and that IL-6 increases osteoclast precursor recruitment through activating hematopoietic progenitor cells [**33**]. In the present study, however, the MR16-1 treatment did not affect the frequencies of peripheral monocytes in the blood, suggesting that the mechanisms of growth and differentiation of osteoclast progenitors are more complex.

IL-6 signal is mediated through a protein complex including the membrane-bound, non-signaling α-receptor subunit (IL-6R) and two signal-transducing gp130 subunits [**33**]. IL-6R is predominantly expressed on hepatocytes, myeloid derived cells, and some lymphocytes, while gp130 is expressed ubiquitously [**34**]. IL-6 transduces signals via membrane-bound IL-6R and signal-transducing gp130 subunits; however, IL-6 signals can also be transduced via soluble IL-6R (sIL-6R) by the association with ubiquitously expressed gp130 [**34,35**]. Mice with a gain-of-function mutation of gp130 were shown to develop arthritis due to the excess IL-6 signal **[36**], supporting the important role of IL-6 in the pathogenesis of RA. It was reported that, while neither IL-6 nor sIL-6R alone stimulates bone resorption, a combined effect of IL-6 and sIL-6R enhanced bone resorption in association with the up-regulated expression of RANKL in mouse calvaria bone explants [**37**]. Because this effect was blocked by neutralizing antibody against gp130, RANKL-producing cells are suggested to express gp130.

In the previous study, we found that the arthritis-free TNFα-deficient KO1 mice showed the same level of RANKL expression in joint tissues as that found in KO1 mice with overt arthritis [**23**]. This is in contrast to the present findings, in which the RANKL expression level in arthritis-free MR16-1-treated KO1 mice was significantly lower than

the levels in arthritis-prone untreated and rat IgG-treated KO1 mice. Thus, it appears that IL-6, but not TNFα, may transduce signals to enhance the expression level of RANKL. This is consistent with the report showing that IL-6/sIL-6R but not TNFα directly induces RANKL expression in fibroblast-like synoviocytes from RA patients [**38**]. On the other hand, the current as well as our earlier studies [**23**] showed that the expression levels of OPG were markedly down-regulated in arthritis-prone untreated and normal rat IgG-treated KO1 mice. OPG is produced by a variety of cells including osteoblasts, B cells, dendritic cells, and vascular endothelial cells [**39,40**]. Marked decrease in OPG expression in arthritic joints is suggested to be due to the exhaustion of OPG production over extended periods of chronic stimulation by TNFα [**40**]. Consistently, synovial OPG expression levels were increased in anti-TNFα-treated RA patients [**41**] as well as in TNFα-deficient KO1 mice [**23**]. In the present study, MR-16-1 treatment restored the OPG expression in joint tissues. This restoration in mice with the preventive treatment of MR-16-1 may be due to the down-regulated expression of TNFα. Alternatively, IL-6 may contribute to the suppression of OPG expression independent of the effect of TNFα, since Liu et al. [**42**] reported that IL-6 mediates the inhibitory effects on OPG production via the increased secretion of

prostaglandin E2. In our therapeutic model, the TNFα expression levels were comparable between the MR-16-1-treated and untreated KO1 mice; nonetheless ~~however~~, the treated mice showed lower IL-6 and higher OPG expressions in ankle joint tissues than the findings in untreated mice, suggesting that the change in OPG expression in joint tissues may be independent of the effect of TNFα .

In hierarchical relationships within cytokine networks involved in the pathogenesis of RA, a linear model has been proposed, in which TNFα would drive downstream cytokine generation, such as IL-1 and IL-6, sequentially [**43**]. The present study showed that, while the IL-6 signal blockade was ineffective to suppress the ongoing TNFα production, this blockade in the preclinical stages reduced the TNFα expression in joint tissues, suggesting the presence of another pathway of cytokine network. Intriguingly, it has been shown that the serum IL-6 level is increased in tocilizumab-treated RA patients [44]. This increase was suggested to be mainly due to the decreased IL-6R-mediated degradation of IL-6, since IL-6 mRNA expression in synovial biopsy samples was rather decreased after administration of tocilizumab [45]. This is consistent with the finding in the current study that IL-6 mRNA expression level in ankle joint tissues was decreased in MR16-1-treated mice, as compared with that in untreated mice.

However, because the IL-6 mRNA level in mice with the preventive MR16-1 treatment was much lower than that found in mice with the therapeutic treatment, it is likely that the blockade of IL-6 signals in earlier stages of arthritis is more effective to suppress inflammatory cytokines, such as TNFα and IL-6.

Since RA is a chronic autoimmune disease developing under the regulation of multiple susceptibility factors, it is plausible that different sets of proinflammatory cytokines/chemokines may be involved in the pathogenesis of RA particularly in different clinical and pathological stages. As the same RA phenotype may develop under different mechanisms, it is also plausible that different sets of proinflammatory cytokines/chemokines may cause phenotypically similar clinical subsets of RA. Our arthritis-prone KO1 mouse model, as well as others, is one of the most useful mouse models for clarifying the mechanisms of ongoing crosstalk of causative cytokines/chemokines in the pathogenesis of various subsets of RA, in order to search for a tailor-made therapy against this complex chronic autoimmune disease.

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Conflict of interest

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Figure legends

Figure 1 Preventive effect of MR16-1 treatment. (a) The cumulative incidence of arthritis and arthritis score (mean and SE) in untreated (n=15), normal rat IgG-treated (n=15), and MR16-1-treated KO1 (n=15) mice. Statistical significance is shown (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). (b) Representative macroscopic findings of hind paws in untreated, normal rat IgG-treated, and MR16-1-treated KO1 mice at 10 months of age. (c) Representative histopathological changes in finger joints in the three groups of KO1 mice at 10 months of age. Untreated and normal rat IgG-treated KO1 mice show marked synovitis with inflammatory cell infiltration, pannus formation (Pn), TRAP-positive osteoclast generation (arrowhead), and the destruction of cartilages and bones. There are no such changes in MR16-1-treated KO1 mice. Representative results obtained from six female mice in each group. Hematoxylin/eosin and TRAP staining. Bars = 50 μm.

Figure 2 Comparisons of serum levels of RF, and IgG class autoantibodies against CCP and dsDNA among untreated, normal rat IgG-treated, and MR16-1-treated KO1 mice at 8 months of age. The horizontal bar represents the mean level. Statistical significance is

shown (* $P<0.05$, ** $P<0.01$).

Figure 3 Comparisons of mRNA expression levels of (a) RANKL and OPG, (b) RANKL/OPG ratio, (c) MCP-1, RANTES, and CX3CL1, (d) IL-6, TNFα, and IFNγ, and (e) Foxp3 and RORγt in ankle joints by quantitative real-time PCR analysis among untreated, normal rat IgG-treated, and MR16-1-treated KO1 mice at 8~9 months of age. The value of normal B6 mice was designated as 1, and values of each of the three groups of mice were evaluated as fold change compared with the value in B6 mice. Data are shown as mean + SEM of four mice from each group. Statistical significance is shown (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Figure 4 Therapeutic effect of MR16-1 treatment. (a) Seven-month-old KO1 mice with arthritis score 1 or 2 were treated or untreated with MR16-1 during 8 weeks. The sequencial changes of arthritis score are shown. (b)~(e) Comparisons of mRNA expression levels of (b) RANKL and OPG, (c) RANKL/OPG ratio, (d) MCP-1, and (e) IL-6 and TNFα in ankle joints by quantitative real-time PCR analysis between untreated and MR16-1-treated KO1 mice at 9 months of age. The values were evaluated as fold

change compared with the value in B6 mice, as shown in Fig. 3. Data are shown as mean + SEM of four mice from each group. Statistical significance is shown (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 1　Spleen weight, splenic lymphocyte subpopulations, and peripheral monocyte frequencies in untreated, normal rat IgG-treated, and MR16-1-treated KO1 mice.[a]

	none	rat IgG	MR16.1
Spleen weight (gm)	0.18±0.03	0.19±0.04	0.18±0.05
Spleen cell populations (%)			
$B220^+$B cells/total cells	60.0±4.8	63.9±0.8	66.1±2.9
$CD69^+B220^+$ B cells/total B cells	5.6±0.3	6.0±1.2	2.2±0.6[b]
PNA^+B220^+ B cells/total B cells	1.9±0.8	2.3±0.5	2.5±0.2
$CD138^+$plasma cells/total cells	1.1±0.6	0.7±0.2	1.0±0.2
$CD3^+$T cells/total cells	20.9±6.0	20.0±0.2	20.2±3.7
$CD69^+CD3^+$ T cells/total T cells	29.3±2.5	24.8±2.2	24.6±5.8
$CD4^+$ T cells/total cells	17.5±0.4	16.2±0.8	11.9±0.7[b]
$CD8^+$ T cells/total cells	3.9±0.4	5.2±0.3	7.9±2.9
CD4/CD8 ratio	4.6±0.6	3.1±0.0	1.9±0.4[c]
$CD25^+Foxp3^+$ T cells/$CD4^+$ T cells	21.3±1.4	23.2±2.8	24.2±3.5
Peripheral $CD11b^+$ monocytes (%)	54.3±11.4	50.5±11.9	56.9±7.3

[a]Values are the mean ± SEM of at least 6 female mice aged 9-10 months.

[b]Differences were statistically significant versus KO1 ($P < 0.01$) and versus normal rat IgG-treated KO1 ($P < 0.05$).

[c]Differences were statistically significant versus KO1 ($P < 0.05$).

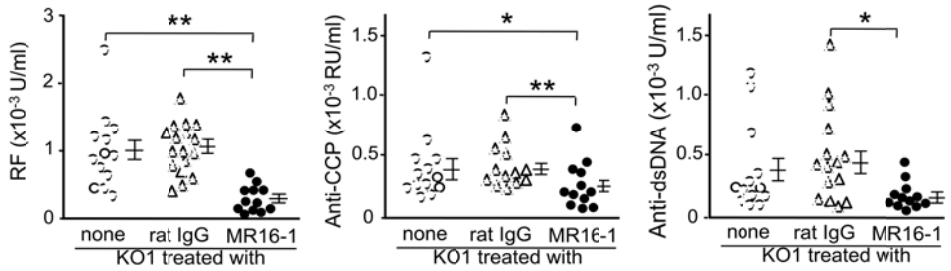
Table 2 Frequencies of $CD4^+$ T cell subsets in *in vitro* stimulated spleen cells in untreated and MR16-1-treated KO1 mice.[a]

	none	MR16.1
$TNF\alpha^+$ T cells/total $CD4^+$ T cells	59.1±2.0	50.3±1.0[b]
$IFN\gamma^+$ T cells/total $CD4^+$ T cells	54.3±4.4	55.1±1.6
IL-4^+ T cells/total $CD4^+$ T cells	0.67±0.2	0.38±0.1
IL-17^+ T cells/total $CD4^+$ T cells	0.23±0.05	0.23±0.05

[a]Values are the mean ± SEM of at least 4 female mice aged 9 months.

[b]Differences were statistically significant versus KO1 ($P < 0.05$).

Fig. 2


RF (x10-3 U/ml)
3
2
1
0
**
**
none
rat IgG
MR16-1
KO1 treated with
Anti-CCP (x10-3 RU/ml)
1.5
1.0
0.5
0
*
**
none
rat IgG
MR16-1
KO1 treated with
Anti-dsDNA (x10-3 U/ml)
1.5
1.0
0.5
0
*
none
rat IgG
MR16-1
KO1 treated with