Obesity does not diminish the efficacy of IL-6 signalling blockade in mice with collagen-induced arthritis

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Abstract

Objective

Obese rheumatoid arthritis patients often have higher disease activity and a poorer response to treatment than do non-obese patients. The present study aims to clarify the influence of obesity on the action of IL-6 and to evaluate the efficacy of IL-6 signalling blockade in arthritis with obesity.

Method

Mice were fed a high-fat diet for 5 weeks, and the influence of this diet on macrophages and type II collagen-induced arthritis was investigated.

Results

The mice fed the high-fat diet showed greater expression of macrophage marker F4/80, not only in subcutaneous fat but also in knee synovium and the calcaneal region, than did the mice fed a normal diet. Furthermore, macrophages isolated from mice on the high-fat diet tended to show higher expression of cyclooxygenase-2 following IL-6 stimulation than did macrophages from mice fed the normal diet. Moreover, mice fed the normal or high-fat diet were immunised with type II collagen, and were treated with anti-mouse IL-6 receptor antibody (MR16-1). The anti-arthritis effect of MR16-1 was not reduced in mice fed the high-fat diet compared to mice fed the normal diet (inhibition ratio: 87% vs. 62%). Furthermore, at the peak of arthritis, cyclooxygenase-2 expression in the calcaneal region of mice fed the high-fat diet was higher than that in the mice fed the normal diet.

Conclusion

These results suggested that a high-fat diet induces inflammatory changes in the synovium. We demonstrated that IL-6 signalling blockade by an anti-IL-6 receptor antibody can be effective in treating arthritis, even with obesity.

Key words

collagen-induced arthritis, interleukin-6, obesity, rheumatoid arthritis, tocilizumab

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease mainly affecting the synovium of the joints and surrounding tissues. The inflammation in RA is caused by overproduction of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α), and by activation of inflammatory cells such as macrophages and proliferation of synovial tissue, ultimately leading to joint destruction. Inhibitors of IL-6 signalling, such as tocilizumab, and inhibitors of TNF-α signalling, such as infliximab, are very effective against RA and are now widely used (1, 2).

Nowadays, obesity is considered a state of chronic low-level inflammation. Hypertrophied adipose tissue produces adipokines such as adiponectin, leptin, and resistin. IL-6 and TNF-α are also produced by adipocytes and by infiltrating macrophages. Because of this chronic inflammatory status, obesity constitutes a risk factor in many types of disease, such as diabetes, atherosclerosis, cancer, and osteoarthritis (3–6). However, the influence of obesity on RA is not yet completely clear.

It is reported that the influence of obesity on RA patients differs in some ways from the influence of obesity on non-RA patients. For example, in RA patients, fat mass does not influence endothelial function, a marker of cardiovascular risk, whereas a negative correlation between fat mass and endothelial function is observed in non-RA patients (7). In addition, unlike with other diseases, there are conflicting findings regarding the influence of obesity on RA: Some studies have reported higher disease activity in obese RA patients than in non-obese RA patients (8, 9), but this finding is not confirmed by others (10–12). Furthermore, it is reported that even though obese RA patients have more active symptoms than do non-obese RA patients, they show slower bone damage progression, suggesting that high body mass index has a protective effect on the destruction of small joints in RA (13). Obesity has also been found to exert a negative influence on the therapeutic effects of anti-TNF-α treatment of RA (9, 10, 14).

Although the pathophysiological mechanisms underlying how obesity influences this response to anti-TNF-α treatment are not yet clear, the fact that some treatments for RA are adversely affected by obesity raises the clinically important question of whether or not the efficacy of other treatments is also reduced in obese RA patients. Pers *et al*. reported that there was no significant association between body mass index and the response to tocilizumab during 6 months of treatment (12). We speculated that there would be some differences between the influence of IL-6 and that of TNF-α on RA with obesity, because IL-6 and TNF-α have different influences on the lipid profile. TNF-α inhibitors are reported to increase high-density lipoproteins whereas tocilizumab induces elevation of low-density lipoproteins (15).

The present study aimed to clarify the influence of obesity on the action of IL-6, and to evaluate the efficacy of IL-6 signalling blockade in arthritis with obesity. For this purpose, we used an obese collagen-induced arthritis (CIA) mouse model, which is reported as a model of obese RA (16, 17). In this model, arthritis is induced by type II collagen (CII) and obesity is induced by a high-fat diet.

Materials and methods

Test agents

Rat anti-mouse IL-6 receptor monoclonal antibody, MR16-1, was prepared using a hybridoma established in our laboratory (18).

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd.

Three-week-old male DBA/1 mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), and 114 mice in total were used in this study (details are included in the figure legends). All mice were housed in a specific pathogen-free environment under controlled conditions (temperature, 20°C–26°C; humidity, 30%–75%; light/dark cycle, 12 h/12h). Chlorinated water and irradiated food were provid-

ed ad libitum. All mice were allowed to acclimatise and recover from shipping-related stress for at least 7 days prior to the study. At four weeks old, the mice were fed either a normal diet (CE-2, 344.9 kcal/100 g, 4.6% energy as fat; CLEA Japan, Tokyo, Japan) or a high-fat diet (F2HFHSD, 481 kcal/100g, 54.5% energy as fat; Oriental Yeast, Tokyo, Japan) until the end of the experiment.

Induction of CIA and treatment regimen

CIA was induced as previously described (19). In brief, 9- or 11-week-old mice (after 5 or 7 weeks on the high-fat diet) were immunised intradermally (Day 0) at the base of the tail with 200 μg (50 μL of 4 mg/mL) bovine type II collagen (CII; Collagen Research Center, Tokyo, Japan) emulsified with an equal volume of complete adjuvant H37Ra (DIFCO, Detroit, MI, USA). Three weeks later (Day 21), mice received a booster immunisation in the same manner. Clinical symptoms of arthritis were evaluated visually and mice were assigned an arthritis score on a scale of 0 to 4 for each limb (0, "no change"; 0.5, "swelling and erythema of one digit"; 1, "swelling and erythema of two or more digits"; 2, "mild swelling and erythema of the limb"; 3, "gross swelling and erythema of the limb"; and 4, "gross deformity and inability to use the limb". Therefore the maximum prospective score per mouse is 16) (20). MR16-1 was intraperitoneally administered at a dose of 8 mg twice (Day 0 and 21).

Extraction of total RNA in subcutaneous fat, knee synovium, and the calcaneal region

Subcutaneous fat, knee synovium, and tissue from the calcaneal region were excised from mice fed a high-fat diet or normal diet for 5 weeks, and immersed in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA, USA) and stored at -80°C until total RNA extraction. Total RNA was extracted using an RNeasy Fibrous Tissue Kit (Qiagen) and RNeasy Lipid Tissue Kit (Qiagen) according to the manufacturer's instructions.

Isolation of primary macrophages and extraction of total RNA from macrophages

Ice-cold phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 1 mM ethylenediaminetetraacetic acid was injected with an 18G needle into the peritoneal cavity of isoflurane-anesthetised mice that had been fed a high-fat diet or normal diet for 7 weeks (before induction of CIA). The peritoneum was gently massaged to dislodge any attached cells into the PBS, and cells were collected while moving the tip of the needle carefully to avoid clogging with fat tissue or other organs. Cells collected from 2 animals on each diet were pooled and suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum. Macrophages were seeded on 24-well plates at 2×10^5 cells/well in RPMI-1640 supplemented with 10% fetal bovine serum. The cells were stimulated with 10 ng/mL of mouse IL-6 (R&D Systems, Minneapolis, MN, USA) for 24 h. Total RNA was extracted using an RNeasy kit (Qiagen) according to the manufacturer's instructions.

Measurement of mRNA expressions

cDNA was synthesised from total RNA with an Omniscript RT kit (Qiagen) using random 9-mer primers (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed by running a TaqMan gene expression assay (Life Technologies, Gaithersburg, MD, USA) targeting mouse IL-6, TNF-α, F4/80, cyclooxygenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on an ABI PRISM 7500 system (Life Technologies) according to the manufacturer's instructions. All primers were obtained from Life Technologies.

Measurement of serum amyloid A and IL-6 levels in serum

Serum samples were collected on Day 24 from the first CII immunisation, at which point symptoms of arthritis (as measured by arthritis score) had not yet developed, and the concentrations of serum amyloid A (SAA) and IL-6 were measured by mouse SAA ELISA kit (Life Technologies) and mouse IL-6 Quantikine ELISA Kit (R&D Systems), respectively.

Statistical analysis

Statistical significances were estimated by unpaired *t*-test or Wilcoxon's test for comparison of two groups using the JMP 11 software package (SAS institute Japan, Tokyo, Japan) with significance level set to 5%.

Results

Effect of IL-6 on F4/80 and COX-2 expression in primary macrophages

Mice fed the high-fat diet for 5 weeks showed a significant increase in macrophage accumulation, as demonstrated by increases in F4/80 expression in subcutaneous fat, knee synovium, and tissue from the calcaneal region (Fig. 1A). Inflammatory changes induced by the high-fat diet were also demonstrated by the increases in IL-6 and TNF-α mRNA in the knee synovium (Fig. 1B). The expression of F4/80 in primary macrophages did not differ according to the diet and did not change with IL-6 stimulation (Fig. 1C). On the other hand, IL-6 stimulation significantly increased the expression of COX-2 in macrophages from mice fed the normal diet and in macrophages from mice fed the high-fat diet fed, and the response tended to be higher in macrophages from mice fed the high-fat diet than in macrophages from mice fed the normal diet (Fig. 1D).

Anti-arthritic effect of MR16-1 in CIA mice

Excess body weight gain was induced by the high-fat diet. Although body weight decreased with the development of arthritis (Day 33, last point of Figure 2A), in every group throughout the experiment, the body weight of mice fed the high-fat diet was higher than that of mice fed the normal diet (Fig. 2A).

Arthritis scores of CIA mice fed the high-fat diet were slightly higher than those of mice fed the normal diet, but the difference was not significant. MR16-1 significantly inhibited the arthritis scores in mice fed both diets, and the anti-arthritis effect of MR16-1 treatment was not reduced by the high-

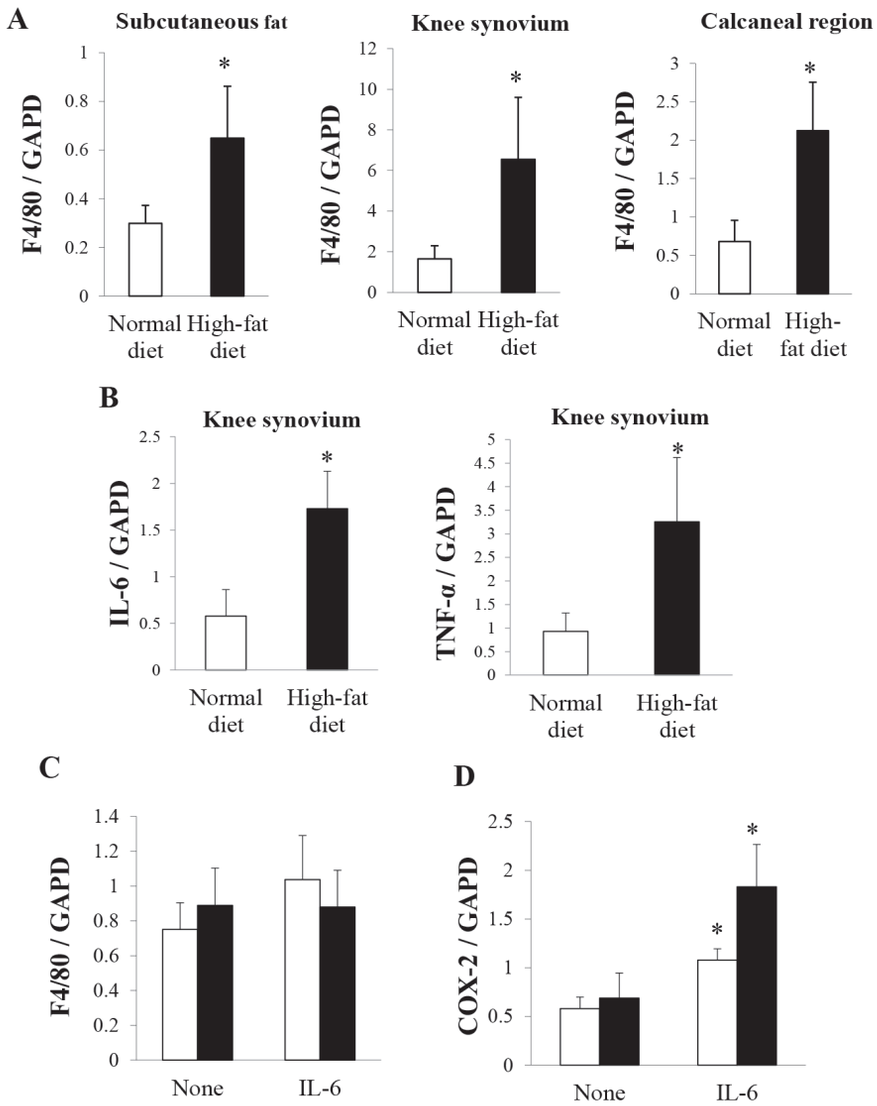

Fig. 1. Effect of IL-6 on F4/80 and COX-2 expression in primary macrophages. Four-week-old DBA/1 mice were fed either a normal diet (white bars) or a high-fat diet (black bars) for 5 weeks (**A, B**) or 7 weeks (**C, D**). F4/80 levels in the subcutaneous fat, knee synovium, and calcaneal region were measured by real-time PCR (**A**). IL-6 and TNF-α levels in knee synovium were measured by real-time PCR (**B**). Each column and vertical line indicates mean + SD of 5 animals. Statistical significance was analysed by unpaired *t*-test (*: $p<0.05$ *vs*. normal diet). Primary mouse macrophages were cultured with or without IL-6 (10 ng/mL). Twenty-four hours later, levels of F4/80 (**C**) and COX-2 (**D**) were measured by real-time PCR. Each column indicates the mean + SD of triplicate cultures. Statistical significance was analysed by unpaired *t*-test (*: $p<0.05$ *vs*. none).

fat diet (inhibition ratio: high-fat group, 87%; normal group, 62%) (Fig. 2B).

Inhibition of IL-6 signalling by MR16-1 in CIA mice fed the high-fat diet

CII immunisation induced an increase in SAA and IL-6 levels at Day 24, both in mice fed the high-fat diet and in those fed the normal diet. The SAA level in control mice (*i.e*. CIA mice without MR16-1) fed the high-fat diet was higher than that in control mice fed the normal diet (Fig. 3A). Both in mice fed the high-fat diet and in those fed the normal diet, MR16-1 completely inhibited the SAA levels and significantly increased serum IL-6 levels (Fig. 3A-B).

Effect of MR16-1 on COX-2 expression in the calcaneal region of CIA mice fed the high-fat diet

CIA induced COX-2 expression in the calcaneal region at Day 33, both in mice fed the high-fat diet and in those fed the normal diet (Fig. 4A). COX-2 expression was well correlated with arthritis score (Fig. 4B). The COX-2 expression of control mice fed the high-fat diet was higher than that of control mice fed the normal diet. In both diet groups, MR16-1 significantly inhibited COX-2 expression (Fig. 4A).

Discussion

A high-fat diet induced an increase in F4/80 expression, not only in subcutaneous fat but also in knee synovium and the calcaneal region (Fig. 1A). These results suggested that the high-fat diet enhanced accumulation of macrophages in those tissues because the expression of F4/80 in macrophages isolated from mice fed the high-fat diet was at the same level as that in macrophages isolated from mice fed the normal diet (Fig. 1C).

It has been previously reported that a high-fat diet induces adipocyte hypertrophy and an increase in macrophage infiltration into adipose tissue in mice (21, 22) and humans (21). However, the results of the current study are the first to demonstrate an increase in macrophage accumulation in knee synovium and the calcaneal region accompanying the obesity induced by a high-fat diet in mice. The inflammatory changes induced in the calcaneal region by the high-fat diet were also confirmed by the increased expression of inflammatory cytokines. Furthermore, macrophages isolated from obese mice reacted more sensitively to IL-6; stimulation with IL-6 induced a larger amount of COX-2 in macrophages from obese mice than in macrophages from mice of normal weight (Fig. 1D). Although the reason for the change in IL-6 response in macrophages is not yet clear, changes in the ratio of macrophage subtypes (M1 and M2) might be involved. Further investigation is required to clarify this point.

Next, we studied the influence of the high-fat diet on the arthritis model. Arthritis scores of obese mice fed the high-fat diet tended to be higher (but not significantly higher) than those of mice fed the normal diet in our study (Fig. 2B). Several reports have described the influence of a high-fat diet in the arthritis model. Jhun *et al*. report-

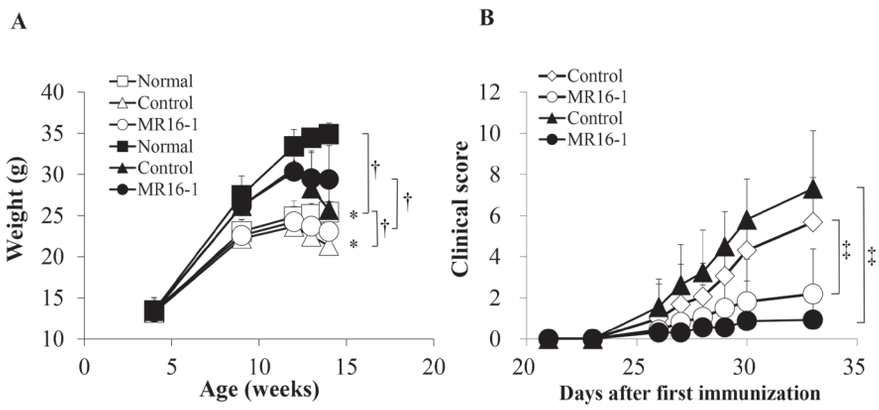

Fig. 2. Anti-arthritic effects of MR16-1 in CIA mice fed a high-fat diet. Four-week-old DBA/1 mice were fed either a normal diet (white symbols) or a high-fat diet (black symbols). After 5 weeks, mice were immunised with 200 μg of CII (Day 0, 9-week-old mice). Three weeks later (Day 21, 12-week-old mice), mice received a booster immunisation in the same manner. MR16-1 was administered at a dose of 8 mg twice (Day 0 and 21). Time course of body weight (**A**) and arthritis scores (**B**). Arthritis scores were assessed as described in *Materials and Methods*. Data points indicate mean + SD of 4 animals (normal groups) or 8 animals (CIA groups). Statistical significance of differences in body weight was analysed by unpaired *t*-test (*: $p<0.05$, Normal *vs*. Control of each diet at Day 33 [14-week-old mice, last point]; †: $p<0.05$, normal diet *vs*. high-fat diet of each treatment as AUC of body weight throughout the experimental period), and statistical significance of differences in arthritis score was analysed by Wilcoxon's test (‡: $p<0.05$, Control *vs*. MR16-1 of each diet as AUC of arthritis score through the experimental period).

ed that a high-fat diet induced a slight but significant increase in the arthritis score in the CIA model using C57BL/6 mice (16). Kim *et al*. reported that, in a CIA model using DBA1 mice, a high-fat diet induced significantly earlier disease onset but the arthritis score in the late phase did not differ between mice fed a normal diet and those fed a high-fat diet (23). The influence of a high-fat diet on arthritis might differ depending on experimental conditions such as mouse strain, constituents of the diet, or duration of observation. Although we could not demonstrate a significant increase in arthritis score by feeding a high-fat diet, a significant increase in inflammation was demonstrated as an increase in SAA in our model of CIA in mice with obesity. This change in inflammation (measured 3 days after the second immunisation, Day 24) antedated the appearance of arthritis, and even after onset of arthritis, inflammation in the calcaneal region, as demonstrated by COX-2 expression, was higher in mice fed the high-fat diet than in mice fed the normal diet (Fig. 4).

The anti-arthritis effect of MR16-1 was not reduced in mice fed the high-fat diet compared with mice fed the normal diet (Fig. 2B, mean inhibition ratio: 87% *vs*. 62%). As evidenced by the complete inhibition of SAA (Fig. 3A), MR16-1 strongly inhibited IL-6 signalling. MR16-1 might be slightly more effective in mice fed the high-fat diet than in mice fed the normal

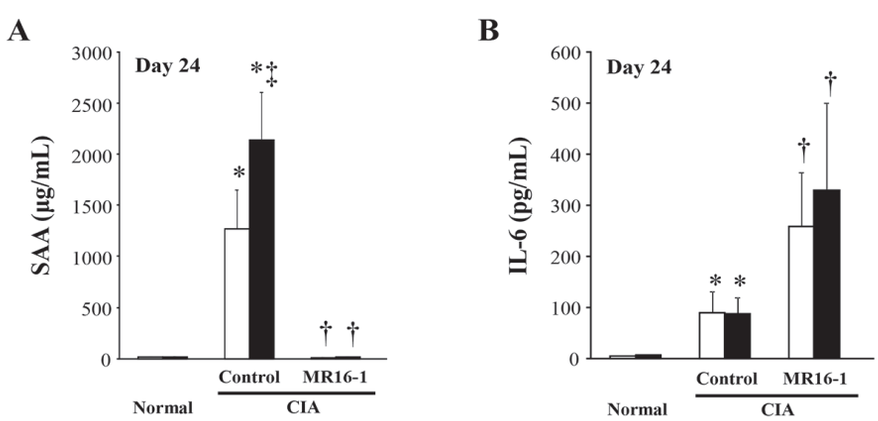

Fig. 3. Inhibition of IL-6 signalling by MR16-1 in CIA mice fed a high-fat diet. CIA model mice were prepared by the same protocol indicated in Figure 2. Levels of SAA **(A)** and IL-6 **(B)** were measured by ELISA in sera obtained on Day 24. Each column and vertical line indicates mean + SD of 5 animals. Statistical significance of differences between groups was analysed by unpaired *t*-test (*: $p<0.05$ *vs*. Normal; †: $p<0.05$ *vs*. Control; ‡: $p<0.05$ *vs*. normal diet). White and black bars indicate mice fed the normal diet and high-fat diet, respectively.

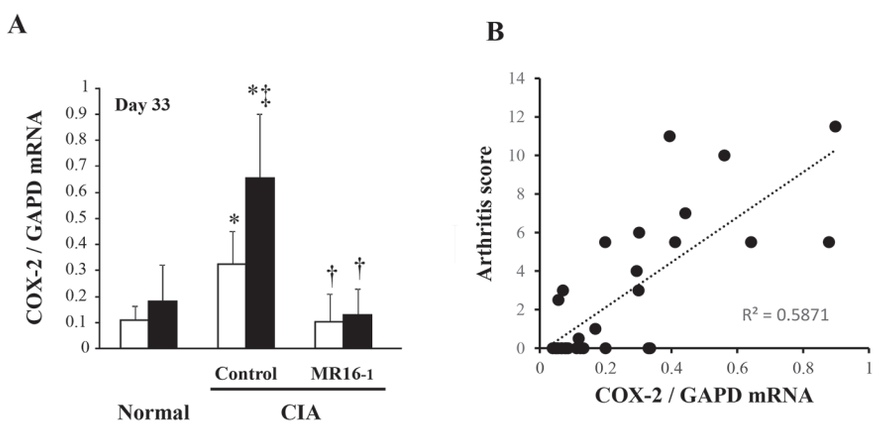

Fig. 4. Effect of MR16-1 on COX-2 expression in the calcaneal region of CIA mice fed a high-fat diet. CIA model mice were prepared by the same protocol indicated in Figure 2. COX-2 levels in the calcaneal region obtained from CIA model mice on Day 33 were measured by real-time PCR. Each column and vertical line indicates mean + SD of 5 animals. White and black bars indicate mice fed the normal and high-fat diet, respectively (**A**). The scatter plot shows the relationship between COX-2 level and arthritis score (**B**). Statistical significance of differences between groups was analysed by unpaired *t*-test (*: $p<0.05$ *vs*. Normal; †: $p<0.05$ *vs*. Control; ‡: $p<0.05$ *vs*. normal diet).

diet because the contribution of IL-6 to inflammation might be increased in this model. Furthermore, MR16-1 was able to exert a sufficient inhibitory effect without an increased dose because the increased contribution of IL-6 was due to the increase in sensitivity to IL-6 and not to increased expression of IL-6. According to the results of a recent retrospective study on tocilizumab in obese RA patients, baseline body mass index did not influence the response to tocilizumab in RA patients (12). MR16-1 treatment significantly increased the level of IL-6 in serum. We previously demonstrated that this phenomenon is due to the inhibition of IL-6 receptor-mediated clearance of IL-6 and not to the induction of IL-6 synthesis or release (24).

A limitation of this study is that we used the increase of F4/80 mRNA as a marker of macrophage accumulation in fat and synovium. F4/80 is a well-known marker for macrophages in mice; however we cannot rule out contamination by non-macrophage cells which also express F4/80, such as dendritic cells or microglia. To confirm the infiltration of macrophages into the synovium, other studies using flow-cytometry or immunohistochemistry will be required.

In conclusion, we demonstrated that in mice fed a high-fat diet, inflammation was increased both with and without induction of CIA, and that this increase in inflammation involved an increase in IL-6 sensitivity in macrophages. IL-6 blockade in mice fed the high-fat diet showed an anti-arthritic effect as strong as that in mice fed the normal diet. It is anticipated that IL-6 receptor inhibitors will become helpful drugs in treating RA in obese patients; however, clinical studies are needed.

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