Original Article

Inhibition of Interleukin-6 Signaling Attenuates Aortitis, Left Ventricular Hypertrophy and Arthritis in Interleukin-1 Receptor Antagonist Deficient Mice

Yoshiko Hada[1,2], Haruhito A. Uchida[1,2], Tomoyuki Mukai[3], Fumiaki Kojima[4], Masashi Yoshida[2,5], Hidemi Takeuchi[1], Yuki Kakio[1], Nozomu Otaka[1,6], Yoshitaka Morita[3], Jun Wada[1]

1. Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
2. Department of Chronic Kidney Disease and Cardiovascular Disease, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
3. Department of Rheumatology, Kawasaki Medical School, Kurashiki, Japan.
4. Department of Pharmacology, Kitasato University School of Allied Health Sciences, Sagamihara, Japan
5. Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
6. Department of Human Resource Development of Dialysis Therapy for Kidney Disease, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.

Running Title:
IL-6 Inhibition on aortitis, LVH, and arthritis (47 characters)

Corresponding author:
Haruhito A. Uchida, M.D., Ph.D.
Department of Chronic Kidney Disease and Cardiovascular Disease,
Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,
2-5-1 Shikata-cho Kita-ku, Okayama, 700-8558, Japan
Phone: (+81) 86-235-7235 Fax: (+81) 86-222-5214

E-mail: hauchida@okayama-u.ac.jp

Key Word: MR16-1, interleukin-1 receptor antagonist, aortitis, arthritis, LV hypertrophy

Word counts; 4664 words

6 figures, 2 tables, and 2 supplemental Figures

Abstract

The aim of this study was to examine whether inhibition of Interleukin (IL)-6 signaling by MR16-1, an IL-6 receptor antibody, attenuates aortitis, cardiac hypertrophy, and arthritis in IL-1 receptor antagonist deficient (IL-1RA KO) mice. Four weeks old mice were intraperitoneally administered with either MR16-1 or non-immune IgG at dosages that were adjusted over time for 5 weeks. These mice were stratified into 4 groups: MR16-1 treatment groups, KO/MR low group (first 2.0 mg, following 0.5 mg/week, n=14) and KO/MR high group (first 4.0 mg, following 2.0 mg/week, n=19) in IL-1RA KO mice, and IgG treatment groups, KO/IgG group (first 2.0 mg, following 1.0 mg/week, n=22) in IL-1RA KO mice, and wild/IgG group (first 2.0 mg, following 1.0 mg/week, n=17) in wild mice. Aortitis, cardiac hypertrophy and arthropathy were histologically analyzed. Sixty-eight % of the KO/IgG group developed aortitis (53% developed severe aortitis). In contrast, only 21% of the KO/MR high group developed mild aortitis, without severe aortitis ($P<0.01$, vs KO/IgG group). Infiltration of inflammatory cells, such as neutrophils, T cells, and macrophages, was frequently observed around aortic sinus of the KO/IgG group. Left ventricle and cardiomyocyte hypertrophy were observed in IL-1RA KO mice. Administration of high dosage of MR16-1 significantly suppressed cardiomyocyte hypertrophy. MR16-1 attenuated the incidence and severity of arthritis in IL-1RA KO mice in a dose-dependent manner. In conclusion, blockade of IL-6 signaling may exert a beneficial effect to attenuate severe aortitis, left ventricle hypertrophy, and arthritis.

(239 words)

Introduction

Interleukin (IL)-1 is a major mediator of inflammation. Binding of IL-1 to IL-1 receptor type 1 subsequently produces many pro-inflammatory cytokines, such as IL-1, IL-6 and tumor necrosis factor (TNF-α),(1) to induce inflammation. Produced IL-1 binds to its receptor following a positive feedback loop to induce more or sustained inflammation. The IL-1 receptor antagonist (IL-1RA) negatively regulates IL-1 signaling by binding and blocking its functional receptors.(2,3) Thus, IL-1RA plays a significant role in development of acute and chronic inflammation.

Previously, IL-1RA deficient (IL-1RA KO) mice were produced to elucidate pathophysiological roles of IL-1.(4) Interestingly, IL-1RA KO mice spontaneously develop several autoimmune diseases, like arthritis,(5) aortitis, (6) and dermatitis(7) that histologically mimic human psoriasis.(8) The most particular phenotype is polyarthropathy that closely mimic rheumatoid arthritis (RA) in humans.(5) Most of IL-1RA KO mice developed arthritis at 14 weeks of age.(9) Histopathology revealed marked synovial and periarticular inflammation in IL-1RA KO mice, with articular erosion caused by invasion of granulation tissues. Therefore, IL-1RA KO mice are recognized useful to investigate arthritis.(5,10,11)

Regarding large vessel vasculitis (LVV) including Takayasu arteritis (TAK) and giant cell arteritis (GCA), the very limited availability of tissue samples from these patients has significantly impeded our progress in understanding the etiology and pathogenesis. In clinical practice, several immunosuppressive agents have been used to treat the patients with TAK and GCA. A few of them can successfully manage the disease activity, however, detail mechanism how these agents can work still remains unclear. Therefore, the creditable animal model for LVV should be extremely essential to figure out the cause of diseases as well as to explore a new therapeutic option. So far, few of the animal models could fully mimic human LVV. Of interest, a half of IL-1RA KO mice develop aortitis at the age of 8 weeks,(9) presenting the immune and pathological features of the human LVV, the accumulation of many inflammatory cells, including T cells, neutrophils, macrophages, and so on. TNF-α is involved in the development of aortitis in this mice model.(9)

In addition, chronic inflammation in association with IL-1/IL-1RA may affect morphology in cardiomyocytes. Continuous activation of gp130, a signal-transducing receptor component for IL-6-related cytokines, causes

myocardial hypertrophy in mice.(12) The pressure overload by degenerative aortic valve stenosis induces left ventricular (LV) hypertrophy.(5,8) However, few have reported the morphological change of cardiomyocytes in IL-1 RA KO mice.

Therapeutic monoclonal antibodies provide their effects via binding to specific target molecules, which is expected to demonstrate rare off-target adverse reactions. However, non-clinical evaluation of monoclonal antibodies (mAbs) is often difficult since they may lack reactivity toward orthologous targets in animals.(13,14) Recently, MR16-1 was developed as Rat anti-mouse IL-6 receptor mAb.(15,16) The MR16-1 has been used in many experimental models to assess the effects of blockade of IL-6 signaling.(17-23)

Given these above, we hypothesized that MR16-1 could attenuate inflammation in IL-1 RA KO mice to improve autoimmune disorders. In the present study, we aimed to examine the effect of blockade of IL-6 signaling by MR16-1 on several autoimmune diseases, like aortitis, LV hypertrophy and arthritis, using IL-1 RA KO mice.

Experimental

Animals and MR16-1

IL-1RA KO mice(4) were obtained from the Institute of Medical Science of the University of Tokyo and maintaining in the animal resource center of Okayama University. All the animal experiments were performed to conform to the NIH guidelines (Guide for the care and use of laboratory animals). The experimental protocol was approved by the Ethics Review Committees for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical sciences (OKU-2019293). All mice were maintained in a barrier facility and fed a normal laboratory diet. MR16-1 was kindly gifted from Chugai Pharmaceutical, Tokyo, Japan.

Experimental Design

Both male and female mice used in this study were 4 weeks old with more than 10 g in body weight. Mice were stratified into 4 groups: MR16-1 low dose (KO/MR low group: first 2.0 mg, following 0.5 mg/week; n = 14), MR16-1 high dose (KO/MR high group: first 4.0 mg, following 2.0 mg/week; n = 20) in IL-1RA KO mice, IgG (KO/IgG group: first 2.0 mg, following 1.0 mg/week; n = 22) in IL-1RA KO mice, and IgG (wild/IgG group: first 2.0 mg, following 1.0 mg/week; n = 19) in wild type mice. All mice were administered intraperitoneally with either MR16-1 or IgG once a week from 4 weeks old for 5 weeks. After anesthetizing the mice with isoflurane (1.5 - 3.0 %, flow rate of 200 - 300 mL/min, inhalation), their tissues and plasma were harvested at 9 weeks old.

Blood Pressure, Heart Rate, Body and Heart Weight Measurement

Systolic blood pressure and pulse rate were measured by sphygmomanometry using a tail cuff system (BP-98A, Softron) following a published protocol.(24) Conscious mice were introduced into a small holder mounted on a thermostatically controlled warming plate and maintained at 37°C during measurement. Body and heart weights of the study mice were also measured.

Histological and in vivo Evaluation for Aortitis

The aortic valve was fixed in 10 % phosphate-buffered formalin and the cryosections were obtained. Sections (10 μm) were stained with hematoxylin eosin (HE) and masson trichrome. The severity of aortitis was graded on a

scale of 0 to 3 by the degree of inflammation near the aortic valve, as described previously (9); score 0 = normal and no infiltration; score 1 = infiltration and loss of elastic lamellae over less than one third of the media of the aortic sinus; score 2 = loss in one third to two thirds of the aortic sinus; score 3 = loss over more than two thirds of the aortic sinus. Severity of the inflammation around aortic valve was evaluated in a blinded manner by two independent observers.

Immunostaining

Immunohistochemical staining was performed to detect T cell, B cell, neutrophil, and macrophage using a CD3ε antibody (Cell Signaling Technology, Cat.No.99940), B220 antibody (BD Biosciences, Cat.No.557390), Gr-1 antibody (Invitrogen, Cat.No.14-5931-81), and a CD68 antibody (SEROTEC, Cat.No.MCA1957), respectively. Reactivity of the antibodies with tissue antigens was detected using AEC and ImmPACT AEC HRP Substrate (Vector Laboratories) as described previously.(25)

Echocardiography

To examine heart function, transthoracic echocardiography on mice (9 weeks old) was performed without anesthesia by TOSHIBA Xario XG SSA-680A. Left ventricular posterior wall thickness at end diastole was measured via the M-mode of left ventricular parasternal long-axis view. LV diameter at end diastole (LVDd) and at end systole (LVDs) was also measured at same view. Fraction shortening (FS) was calculated by following formula: FS = (LVDd – LVDs) / LVDd x 100.

Measurement of the Size of a Cell

The LV wall including papillary muscles were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections (10 μm) were stained with HE. The cross-sectional area of 100 cardiomyocytes in the LV wall per sample was measured one by one using Image J, then averaged all measurements to determine the representative size of a cell for each mouse.

Plasma Cytokine Levels

Plasma concentrations of proinflammatory cytokine were determined by The Bio-Plex Pro Mouse Cytokine Th17 Panel A 6-Plex Immunoassay (BIO-RAD #M 6000007NY).

Western Blotting

Whole dissected ankles were homogenised in lysisbuffer (Cell Signaling, Cat. No. 9803) containing protease inhibitors by the bone crusher.(26) Each sample was loaded onto 10% SDS-PAGE and transferred to polyvinylidene fluoride membrane, immunoblotted with primary antibodies (p-STAT3; Cell Signaling Cat. No. 9145, STAT3; Cell Signaling Cat. No. 30835). Membranes were then incubated with appropriate secondary antibodies, and immune complexes were visualized on chemiluminescence (Merck Millipore, Cat. no. WBLUF0100, Cat. no. WBLUC0100) and quantified using a General Electric Imager (GE Healthcare, LAS 4000 mini).(24,25)

Macroscopic Evaluation for Arthritis

All mice were examined weekly for 5 weeks. Severity of arthritis was assessed by measuring hind paw thickness with calipers to quantitate edema, determined the incidence of joint rate in front and hind paws (25 % for each paw and the maximum incidence being 100 %), and the degree of arthritis was evaluated on a scale of 0 (no inflammation) to 3 (severely inflamed) for each paw (the maximum score being 12).(27) The degree of arthritis was evaluated in a blinded manner by four independent observers. The incidence of arthritis was determined as the arthritis score of 1 or more.

Histological and in vivo Evaluation for Arthritis

Joints were fixed in 4 % paraformaldehyde, decalcified in 10 % EDTA-4Na, and embedded in paraffin. Sections (5 μm) were stained with HE and tartrate-resistant acid phosphatase (TRAP).(28) Severity of the inflammation and erosion around talus bones and cuneiform bones was evaluated in a blinded manner by four independent observers using scoring systems. In the HE staining, the severity of inflammation was determined by the area of inflammatory cells infiltrates; score 0 = no significant inflammatory cell infiltrates, score 1 = mild diffuse inflammatory cell infiltrates, score 2 = moderate inflammatory cell infiltrates, score 3 = marked inflammatory cell infiltrates. The severity of erosion was determined by the area of erosion; score 0 = no erosion, score 1 = mild erosion, score 2 = moderate erosion, score 3 = severe erosion. In TRAP staining, the number of osteoclasts around talus bones and cuneiform bones was counted.

Statistics

All statistical analyses were performed using Sigma Plot v14.0 (Systat Software Inc. California, USA). Data are presented as mean ± standard deviation or standard error of the mean where appropriate. Statistical significance among multiple groups was assessed by one-way analysis of variance followed by Holm-Sidak post hoc test or Student-Newman-Keuls post hoc test. Severity of aortitis was analyzed by Fisher Exact test. A P value < 0.05 was considered statistically significant.

Results

Hemodynamics and Weight

No significant difference was found in systolic blood pressure and heart rate among 4 groups (Table 1). (9) IL-1RA KO mice developed normally before weaning at 4 weeks old and were fertile. However, their growth became retarded at 6-to-9 weeks old; a significant difference was found between wild and KO in IL-1RA genotype regardless of the treatment at 9 weeks old (wild vs KO, $P < 0.05$). These observations suggest a beneficial role for IL-1RA in growth and homeostasis. (4)

MR16-1 Attenuated Aortitis in IL-1RA KO Mice

To investigate development and severity of aortitis, we performed HE (Figure 1Aa-c) and masson trichrome (Supplemental Figure 1A-D) staining of the aortic valve sections. Figure 1.A showed a representative photograph of each score for arterial inflammation around the aortic sinus of IL-1RA KO mice. (9) No mice in the wild/IgG group developed aortitis. In the KO/IgG group, the percentage for score 0, 1, 2, and 3 were 32, 32, 9, and 27 %, respectively. In the KO/MR low group, the percentage for score 0, 1, 2, and 3 were 57, 36, 7, and 0 %, respectively. In the KO/MR high group, score 0 was 79 %, the remainder was score 1 (Figure 1B). Of the mice with aortitis in the KO/IgG group, 54 % developed moderate-severe aortitis. In contrast, no mice in the KO/MR high group developed moderate-severe aortitis. Two out of eight mice with severe aortitis of the KO/IgG group died. This group displayed serious phenotype of aortitis ($P = 0.00414$, Figure 1C).

Innate Inflammatory Cells Infiltrate into the Vessel Wall in Aortic Lesions

To evaluate which types of immune cells are accumulated into aortic lesions in aortitis, the aortic valve sections were stained with antibodies against several cell type-specific markers. A large number of CD3 positive cells (Figure 2Ab, Af) and many Gr-1 positive cells (Figure 2Bb, Bf) were dominantly observed, smaller populations of CD68 positive cells (Figure 2Cb, Cf) and B220 positive cells (Supplemental Figure 2B, b) in the aortic lesions of the KO/IgG group. Infiltration of several types of inflammatory cells was observed in the KO/IgG group. In the MR treatment groups as well as the wild/IgG group, few infiltrations of inflammatory cells was observed. Thus, MR16-1 significantly

suppressed the infiltration of inflammatory cells (Figure 2, Supplemental Figure 2).

Heart Weight

Similarly to body weight, the KO/IgG and the KO/MR high groups also showed a small but significant decrease in heart weight (vs the wild/IgG group). However, no significant difference was found in heart weight-to-body weight ratio among 4 groups (Table 2).(9)

Development of Cardiac Hypertrophy in IL-1RA KO Mice

Aortic valve plays an essential role in heart function. Since arterial inflammation in IL-1RA KO mice occurs specifically in the aortic sinus, we performed echocardiograms of 4 groups to examine aortic valve function. The representative echocardiogram of each group was shown (Figure 3Aa-d). The thickness of the LV posterior wall in the KO/IgG and the KO/MR low groups notably increased compared to that in the wild/IgG group (Figure 3B), but not statistically significant in the KO/MR high group (Figure 3B). FS of the KO/MR low group increased significantly compared to other groups (Figure 3B), however, FS of all groups were within the normal range. Furthermore, to evaluate the morphological changes of cells in LV, heart sections were stained with HE. The representative images of mice of 4 groups were shown (Figure 3Ca-d). The size of cardiomyocytes was significantly larger in the KO/IgG and the KO/MR low group than that in the wild/IgG group (Figure 3D). However, the size of these cells was not significantly different between the wild/IgG and the KO/MR high group (Figure 3D).

Effects of IL-6 inhibition by MR-16

Plasma levels of proinflammatory cytokines were shown (Figure 4A). Administration of MR16-1 significantly increased the plasma concentration of IL-6 compared to the IgG groups, which most likely reflects a release of receptor-bound IL-6 by MR16-1, as previously reported.(29) Namely, this increment of the concentration of IL-6 suggests that MR16-1 enables to inhibit IL-6 signaling. Plasma concentrations of other cytokines did not significantly differ among 4 groups. In order to confirm the inhibitory effect of MR16-1, activation of STAT3, which is a downstream signal of IL-6, was evaluated by western blotting (Figure 4B). STAT3 activation significantly increased in the KO

/ IgG group compared with the wild / IgG group. Treatment with MR16-1 at both low and high concentrations abolished the inhibition of STAT3 activation in the KO/IgG groups compared to wild type/IgG groups. These results demonstrated that MR16-1 inhibits IL-6 signal.

MR16-1 Improved Arthritis in IL-1RA KO Mice

Paw edema, evaluated by paw thickness, significantly increased only in the KO/IgG group compared with the wild/IgG group. In contrast, the paw thickness did not significantly increase by MR16-1 treatment compared to the wild/IgG group (Figure 5A).

Next, the arthritis score was markedly higher in the KO/IgG group and the KO/MR low group than the wild/IgG groups. However, the treatment with MR16-1 at high dosage significantly decreased the arthritis score compared with the KO/IgG group and the KO/MR low group (Figure 5B).

Regarding the incidence of joint rate, a significantly increase was observed in the KO/IgG and the KO/MR low groups compared with the wild/IgG group, with 31 % of the joint incidence in the KO/IgG group and 36 % in the KO/MR low group whereas 0 % in the wild/IgG group exhibiting arthritis determined by the final observation. By contrast, the joint incidence did not significantly differ between the wild/IgG and the KO/MR high group, with only 19 % of the incidence of joint rate in the KO/MR high group exhibiting arthritis by the final observation (Figure 5C). Furthermore, the incidence of arthritis in the KO/MR high group was consistently low from 5 to 9 weeks old compared with the KO/IgG and the KO/MR low group. The significant difference was found in the incidence of arthritis between the KO/MR high group, and the KO/IgG group (P = 0.00001) or the KO/MR low group (P = 0.02304). The treatment with MR16-1 suppressed arthritis in dose-dependent manner (Figure 5D).

MR16-1 Histologically Attenuated Arthritis in IL-1RA KO Mice

To analyze the inflamed joints histologically, HE (Figure 6A-D) and TRAP (Figure 6a-d) staining of the ankle joints were performed. The wild/IgG group exhibited no inflammation and bone destruction in any joints (Figure 6A, a). Whereas, the KO/IgG group developed severe inflammatory cell infiltrates (Figure 6B) and exhibited increased osteoclast formation (Figure 6b). The treatment with MR16-1 suppressed inflammatory cell infiltrates and osteoclast formation in dose-dependent manner (Figure 6B-D, b-d). Next, we quantified

the severity of inflammation and bone erosion using histological scoring systems. The arthritis score was significantly higher in the KO groups than the wild/IgG group (Figure 6E). The arthritis score in the KO/MR high group was significantly lower than the KO/IgG group (Figure 6E). The erosion scores were significantly higher in the KO groups than the wild/IgG group (Figure 6F). However, the erosion score was significantly lower in the KO/MR high group than the KO/IgG group, in the similar fashion of arthritis score. To quantitatively evaluate osteoclast formation in the inflamed joints, we counted the number of the sections around talus bones and cuneiform bones stained with TRAP. The number of osteoclasts significantly increased in arthritic joints in the KO/IgG group and the KO/MR low group compared with the wild/IgG group (Figure 6G). The number of osteoclasts in the KO/MR high group improved compared to the KO/IgG group (Figure 6G). These results suggest that increased bone erosion in inflamed joints of mice is associated with enhanced osteoclast formation in IL-1RA KO mice.

Discussion

Recently, IL-6 has gained attention as a therapeutic target for the treatment of several autoimmune disorders. Growing evidence emerged a new therapy using anti-IL-6 receptor antibody for the treatment of RA. In the present study, using IL-1RA KO mice, we found several observations; Firstly, blockade of IL-6 signaling by MR16-1, an IL-6 receptor antibody, attenuated development of severe aortitis as well as arthritis, through suppression of infiltration inflamatory cells. Secondly, IL-1RA KO mice developed LV hypertrophy in association with cardiomyocyte hypertrophy.(8) MR16-1 suppressed both LV and cardiomyocyte hypertrophy.

The previous study has reported that the incidence of aortitis in IL-1RA KO mice was approximately 50 %, furthermore, the incidence and severity increased in IL-6 deficient condition in IL-1RA KO mice.(9) Our study demonstrated that the incidence of aortitis in IL-1RA KO was in agreement with the previous studies, whereas, blockade of IL-6 signaling by MR16-1 suppressed the incidence and severity of aortitis in IL-1RA KO unlike the previous studies. The reason for the discrepancy can be considered several causes. Firstly, it has previously been reported that data obtained from IL-6-deficient mice may not equate to data obtained by IL-6 blockade using neutralizing antibody, because the complete absence of IL-6 in knockout mice has been reported to display hematopoietic defects.(30) Besides, it has been proposed that IL-6 is a pleiotropic cytokine with pro- and anti-inflammatory properties. It turns out that regenerative or anti-inflammatory activities of IL-6 are mediated by classic signaling whereas pro-inflammatory responses of IL-6 are rather mediated by trans-signaling.(31) These anti-inflammatory effects have been attributed to the inhibition of IL-1 and TNF-α production. The induction of IL-1RA synthesis by IL-6, as well as the release of soluble TNF-α receptors into the circulation, may be equally important factors in the modulation of inflammatory responses.(32) It was also reported that IL-6 deficiency induced overproduction of TNF-α in streptococcus pyogenes-infected mice.(33) Moreover, IL-6 present before the onset as well as in the very early phase of concanavalin A-induced hepatitis induced hepatoprotective pathways, whereas continuation of IL-6 production beyond this early phase, by some other pathway, seems to be harmful to hepatocytes.(34) Thus, it is presumed that the TNF-α/IL-6 balance may be a key factor in regulating immune responses.(35)
IL-1β potently induces the expression of adhesion molecules in the endothelial

cells and promotes the recruitment of neutrophils to the site of inflammation. Upon infiltration to the site of inflammation, neutrophils produce soluble IL-6 receptor (sIL-6R) by proteolytic cleavage of the membrane bound form (mbIL-6R). Endothelial cells are subsequently stimulated via IL-6 trans-signaling, converting chemokine expression from CXC chemokines like keratinocyte-derived chemokines, to CC chemokines like MCP-1, consequently resulting in the accumulation of monocytes and T cells. In addition, L-selectin and ICAM-1 mediated tethering of leukocytes are enhanced. While IL-6 induces apoptosis of neutrophils and hence contributes to neutrophil clearance, maintenance of CD4+ TH17 cells is mediated by IL-6 trans-signaling.(31) Thus, it is likely that MR16-1 binding to sIL-6R might suppress inflammatory cell infiltration induced by IL-1. Histologically, the types of inflammatory cells, loss of elastic lamellae in the aortic media, and neovascularization were also frequently observed, demonstrating inflammation. These pathological findings mimic several aspects of TAK in agreement with a previous report.(6) Unfortunately, acquired samples were limited not enough to perform flow cytometry to evaluate detailed quantification of immune cells IL-1RA KO mice may be, in part, a suitable experimental model for the development of new treatments for TAK. It is suggested that blockade of IL-6 signaling could contribute to the treatment of TAK. In clinical setting, inflammatory cells, particularly Th17 and Th1 cells, and cytokines including interferon-γ, TNF-α, IL-6, IL-8, IL-17A and IL-18, increase in patients with TAK.(36-40) Elevated IL-6 concentration is associated with increase in the activity of LVV.(36,37,41) Recently, 2 large clinical trials show that tocilizumab, a humanized anti-interleukin-6 receptor monoclonal antibody, is highly effective in the treatment for the patients with TAK(41) and GCA.(42) These evidences clearly indicate that IL-6 is involved in the development of LVV. Thus, IL-1RA KO mice can be a useful model to investigate the pathophysiology of TAK.

Although lower body weight is a sensitive indicator of illness in mice, no significant difference was found in BW among the KO/IgG group, KO/MR low group, and KO/MR high group in this study. Therefore, we speculate that the cause of weight loss was not only inflammation but also other factors. Since a few of cytokines, including IL-1 and TNF-α, induce anorexia, the retarded growth rates in IL-1 RA KO mice could be due to appetite loss caused by overproduction of IL-1 in the satiety center in the brain.(4) Additionally, LV hypertrophy was observed in IL-1RA KO mice by echocardiograms and histological analysis

although HW/BW was not significantly different. HW/BW is an important indicator of cardiac hypertrophy, but it is greatly affected by the growing season and nutritional status. Thus, it might be better to compare the HW / the tibia ratio.

So far, the precise mechanism to develop LV hypertrophy in these mice has been poorly elucidated. Two potential mechanisms have been suggested. One is, pressure overload by aortic valve stenosis may induce LV hypertrophy.(5,8) The other is, continuous activation of gp130, a signal-transducing receptor component for IL-6-related cytokines, caused myocardial hypertrophy in mice.(12) In our study, the treatment with MR16-1 at high dosage attenuated cardiomyocyte hypertrophy, finally suppressed LV hypertrophy in IL-1RA KO mice. Although hypertrophy is initially a compensatory mechanism, sustained cardiac hypertrophy ultimately leads to decompensated heart failure.(43) Cardiac remodeling involves several molecular pathways, including neurohormonal activation via the sympathoadrenomedullary and renin-angiotensin-aldosterone systems, oxidative stress, and altered energy metabolism: all these pathways can converge on inflammation.(44) Many researchs have been conducted to investigate the progression of cardiac hypertrophy to heart failure,(44-47) however, the mechanism by which this process is suppressed remains unclear. Therefore, it is crucial to clarify the molecular mechanism underlying cardiac hypertrophy and the function of anti-hypertrophic targets. In recent decades, various signaling pathways that mediate the development of hypertrophy have been identified, such as MAPKs, calcineurin/NFAT, and PI3K/AKT pathways. Furthermore, it has been strongly suggested that inflammation plays a critical role in the development of cardiac hypertrophy and heart failure.(48,49) Levels of inflammatory cytokines, such as IL-1α, IL-6, TNF-α, and MCP-1, which promote progression of cardiac hypertrophy and heart failure, increased in the hypertrophic hearts.(50) The effective blockade of these signaling pathways may provide a promising method for inhibiting cardiac hypertrophy and heart failure. Recently, a cytokine called cardiotrophin has been cloned, which acts on cardiomyocytes to cause hypertrophy and whose structure is closely related to, for example, IL-6 and leukemia inhibitory factor.(51) An alternative possibility is that a complex of physiologically existing IL-6 and sIL-6R(52) may be involved in the regulation of myocardium or may stimulate the expression of cardiac hypertrophy-inducing factor. Furthermore, two recent investigations

have demonstrated that deletion of IL-6 attenuates pressure overload-induced LV hypertrophy and dysfunction,(47) that hypoxia-induced mitogenic factor-IL-6 signaling mediates cardiomyocyte-fibroblast crosstalk to promote cardiac hypertrophy and fibrosis.(44) Consequently, IL-6 blockade by tocilizumab may be potentially an alternative therapeutic strategy for the treatment of cardiac hypertrophy-related heart diseases. The present challenge is to identify pharmacological agents that selectively modulate specific signaling pathways and thereby prevent pathological cardiac hypertrophy. To date, no effective drugs have been found to treat cardiac hypertrophy-related molecular changes.(53)

In the current study, MR16-1 attenuated the incidence and severity of arthritis in IL-1RA KO mice in a dose-dependent manner both macroscopically and histologically, as well as its effect on collagen-induced arthritis in monkey,(54) destructive arthritis in McH-lpr/lpr-RA1 mice,(55) and clinical efficacy studies in human.(56) RA is a common autoimmune disease characterized by chronic joint inflammation and progressive bone loss. Since the disease involves multiple genetic and environmental factors, response to treatment varies and achievement of complete remission for all RA patients is difficult.(57) IL-6 is an important factor in the induction of experimental models of several inflammatory diseases, including collagen-induced arthritis and autoimmune inflammatory arthritis.(58-61) Recently, a humanized antibody against both soluble and transmembrane IL-6 receptor, tocilizumab, has launched in clinical practice, exerting outstanding therapeutic efficacy for RA, juvenile idiopathic arthritis, Castleman's disease, and other autoimmune inflammatory diseases. However, like other drugs, tocilizumab is sometimes ineffective in patients with RA,(62,63) due to the complex of the immunological molecular pathways.(64)

IL-6 is involved not only in the activation of the immune system but also in regenerative processes as well as in the regulation of metabolism, for example, in the maintenance of bone homeostasis and many neural functions. It turned out that pro-inflammatory functions of IL-6 could be inhibited by sgp130Fc, which does not affect IL-6 responses via the mbIL-6R. On the other hand, functions of IL-6 such as metabolic control in the liver and regeneration of the epithelium in the intestine seem to be mediated via the mbIL-6R. This view bears important consequences on the therapeutic blockade of IL-6 as a treatment of chronic inflammatory diseases. Patients with such diseases need to be treated for

many years if not for their entire life span. In view of the complex biology of IL-6, long-term global blockade of this cytokine should be carefully considered. Whereas, most IL-6 blocking agents block both classic- and trans-signaling, specific trans-signaling blockade is still under development.(65)

Certainly, under pathological conditions in which Th17 cells trigger an autoimmune disease, the IL-6 amplifier abnormally enhances its signaling; the dysregulated enhancement mediated by this amplifier may be induced by dysregulated activation of NF-κB and STAT3 in endothelial cells, fibroblasts, and so on, through a variety of stochastic environmental and/or genetic factors. (66) Moreover, TNF-α acts in concert with IL-17A to induce IL-6 production. Thus, a number of factors, including such cytokines as TNF-α and other IL-6 family cytokines, Toll-like receptors mediated signaling, and soluble IL-6R, may act upstream of the IL-6 amplifier,(67) which may explain why targeting IL-6 signaling shows therapeutic efficacy of aortitis, cardiomyocyte hypertrophy, and arthritis in our study.

In human, the deficiency of interleukin-1 receptor antagonist (DIRA) is a very rare autosomal recessive autoinflammatory disease first described in 2009. (68,69) DIRA, the deficiency of IL-1RA, results in unopposed pro-inflammatory signaling via IL-1 and the IL-1 receptor (IL-1R1)(68) that can escalate to life-threatening systemic inflammation, systemic inflammatory response syndrome, and death if untreated.(68,70-72) Patients with DIRA present with pustular psoriasis, osteitis, arthritis, CNS vasculitis and so on. (68,69) However, very limited information is available because a total number of the patients with DIRA is pretty small, thus, further data will be required to elucidate the exact clinical manifestation of DIRA. IL-1RA KO mice may be a suitable experimental model for understanding the inflammatory processes and for development of new treatments for DIRA.

In conclusion, the administration of MR16-1 significantly attenuated development of severe aortitis, cardiomyocyte hypertrophy, and arthritis in a dose dependent manner, by blockade of IL-6 signaling. Furthermore, blocking the inflammation signaling pathway including IL-6 may provide a promising method for inhibiting cardiac hypertrophy. Further studies will be needed in order to block specific trans-signaling of IL-6.

Acknowledgment

We would thank Dr. Yoichiro Iwakura (Center for Animal Disease Models, Research Institute for Science and Technology, Tokyo University of Science, Chiba, Japan) for providing IL-1RA KO mice.

Funding
None

Conflict of Interest
Haruhito A. Uchida and Masashi Yoshida belong to the Department of Chronic Kidney Disease and Cardiovascular Disease which is endowed by Kawanishi Holdings, Chugai Pharmaceutical Company, Boehringer Ingelheim and Terumo Corporation. Tomoyuki Mukai and Yoshitaka Morita received scholarship donations from Chugai Pharmaceutical Company. Jun Wada receives speaker honoraria from Astra Zeneca, Daiichi Sankyo, MSD, Novartis, Tanabe Mitsubishi, Taisho Toyama and receives grant support from Baxter, Chugai, Dainippon Sumitomo, Ono, Teijin.

Author Contribution
Yoshiko Hada and Haruhito A. Uchida designed and performed the mouse work, analyzed the data, and drafted the manuscript. Tomoyuki Mukai, Fumiaki Kojima, Masashi Yoshida, Yoshitaka Morita performed the mouse work and analyzed the data. Hedemi Takeuchi, Yuki Kakio, Nozomu Otaka performed the mouse work. Jun Wada reviewed and edited manuscript. All authors contributed to critical revision of the manuscript.

Clinical Perspectives
1. Background as to why the study was undertaken
Previously, IL-6 KO mice in inflammatory mice model failed to demonstrate any protective effect. However, a few of IL-6 receptor antibodies launched in clinical practice and are highly effective on large vessel vasculitis (Takayasu arteritis and Giant cell arteritis). Our primary question was which is true, that is, blockade of IL-6 signaling is effective or not, for inflammatory diseases like large vessel vasculitis.

2. A brief summary of the results

we found that, by injecting an IL-6 receptor blocker (for mice only) into IL-1 receptor antagonist deficient mice (which show severe vasculitis, arthropathy, and myocardial hypertrophy), blockade of IL-6 signaling is effective to suppress these phenotypes.

3. The potential significance of the results to human health and disease

We for the first time proved that blockade of IL-6 signaling by an IL-6 receptor blocker is highly effective on vasculitis in vivo, as well as in human. Moreover, the blocker attenuates not only arthropathy, but also myocardial hypertrophy caused by IL-1-associated severe inflammation. Now we may have a chance to expand the application of this antibody to many other inflammatory diseases.

Figure Legend

Figure 1. Arterial inflammation around the aortic sinus in IL-1RA KO mice.
A, The severity of aortitis was graded on a scale of 0 to 3 by the degree of inflammation near the aortic valve from 8 weeks old mouse, score 0 = normal and no infiltration; score 1 = infiltration and loss of elastic lamellae over less than one third of the media of the aortic sinus; score 2 = loss in one third to two thirds of the aortic sinus; score 3 = loss over more than two thirds of the aortic sinus. Representative photos of hematoxylin eosin stains (a-d) of the aortic sinus are shown. The scale bar indicates 200 μm. B, This panel shows the distribution of the aortitis score in each group (wild/IgG; n = 17, KO/IgG; n = 22, KO/MR low; n = 14, and KO/MR high; n = 19). C, Severity of Aortitis was further categorized into two levels (mild = score 0 and 1, severe = score 2 and 3) in accordance with the severity of aortitis. The effect of MR16-1 treatment was evaluated by comparing the KO/IgG group and the KO/MR high group (P = 0.00414).

Figure 2. Identification of innate immune cells in aortitis lesions.
Representative photos of immunostainings are shown. Wild/IgG mice (Aa, Ae, Ba, Be, Ca, and Ce), KO/IgG mice (Ab, Af, Bb, Bf, Cb, and Cf), KO/MR low mice (Ac, Ag, Bc, Bg, Cc, and Cg), and KO/MR high mice (Ad, Ah, Bd, Bh, Cd, and Ch) were sacrificed at 9 weeks old, and aortic cryosections were obtained. These sections were stained with mAbs against CD3 (Aa - Ah), Gr-1 (Ba - Bh), and CD68 (Ca - Ch), respectively. The scale bar indicates 200 μm (a - d) and 50 μm (e - h).

Figure 3. The left ventricle hypertrophy in IL-1RA KO mice.
A, Representative echocardiographic images are shown in each group, the wild/IgG group (a), the IL-1RA KO/IgG group (b), the IL-1RA KO/MR low group (c), and the IL-1RA KO/MR high group (d) at 9 weeks old. B, The left ventricular posterior wall thickness at end - diastole (LVPWTd) was measured. Fractional shortening (FS) was calculated and expressed as a percentage. The LVPWTds and FS of the mice are graphed for the wild/IgG group (n = 8), the KO/IgG group (n = 11), the KO/MR low group (n = 6) and the KO/MR high group (n = 7), respectively. Values represent the means ± SEM of three experiments.

C, The hematoxylin eosin staining of cardiac ventricles of the wild/IgG mice (a), the IL-1RA KO/IgG group (b), the IL-1RA KO/MR low group (c), and the IL-1RA KO/MR high group (d) at 9 weeks old. D, Cross-sectional area of cardiomyocytes are graphed for the wild/IgG group (n = 8), the KO/IgG group (n = 11), the KO/MR low group (n = 6) and the KO/MR high group (n = 7), respectively. * $P < 0.05$ Values represent the means ± SEM of three experiments.

Figure 4. Effects of IL-6 inhibition by MR16-1.
A, Plasma concentration of proinflammatory cytokines were shown, upper left presents IL-1β, upper right IL-6, middle left IL-10, middle right IL-17A, lower left IFN-γ, and lower right TNFα in the wild/IgG group (n = 19), the KO/IgG group (n = 20), the KO/MR low group (n = 14) and the KO/MR high group (n = 20), respectively. * $P < 0.05$ vs wild/IgG group, B, Protein expression of p-STAT3 and STAT3 in each group. *$P < 0.05$ (n=3) Values represent the means ± SEM of three independent experiments.

Figure 5. Macroscopic observation of arthritis in IL-1RA KO mice.
Time course of paw thickness (A), arthritis scores (B), incidence of joint rate (C) and incidence of arthritis (D) in the wild/IgG group (n = 13), the KO/IgG group (n = 21), the KO/MR low group (n = 14), and the KO/MR high group (n = 20). * $P < 0.05$ vs wild/IgG group, # $P < 0.05$ vs KO/IgG group, $ $P < 0.05$ vs KO/MR low group. All data are expressed as the mean ± SEM.

Figure 6. Histological observation of arthritis in IL-1RA KO mice.
Representative photos of hematoxylin eosin (A - D) and tartrate-resistant acid phosphatase (a - d) staining of the wild/IgG mice (A, a), the KO/IgG mice (B, b), the KO/MR low mice (C, c) and the KO/high mice(D, d). Original magnifications: 40x. The scale bar indicates 200 μm. E, F and G, Histological scores of inflammation and cartilage damage. E, Mean arthritis scores of the wild/IgG group (n = 19), the KO/IgG group (n = 22), the KO/MR low group (n = 14), and the KO/MR high group (n = 20) respectively. Values represent the means ± SEM of three experiments. F, Mean erosion scores of the wild/IgG

group (n = 19), the KO/IgG group (n = 22), the KO/MR low group (n = 14) and the KO/MR high group (n = 20). Values represent the means ± SEM of three experiments. G, The average number of osteoclasts of the wild/IgG group (n = 19), the KO/IgG group (n = 22), the KO/MR low group (n = 14) and the KO/MR high group (n = 20). * $P < 0.05$ Values represent the means ± SEM of three experiments.

Reference

1. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436-44.
2. Carter DB, Deibel MR, Jr., Dunn CJ, Tomich CS, Laborde AL, Slightom JL, et al. Purification, cloning, expression and biological characterization of an interleukin-1 receptor antagonist protein. Nature 1990;344:633-8.
3. Hannum CH, Wilcox CJ, Arend WP, Joslin FG, Dripps DJ, Heimdal PL, et al. Interleukin-1 receptor antagonist activity of a human interleukin-1 inhibitor. Nature 1990;343:336-40.
4. Horai R, Asano M, Sudo K, Kanuka H, Suzuki M, Nishihara M, et al. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 1998;187:1463-75.
5. Horai R, Saijo S, Tanioka H, Nakae S, Sudo K, Okahara A, et al. Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice. J Exp Med 2000;191:313-20.
6. Nicklin MJ, Hughes DE, Barton JL, Ure JM, Duff GW. Arterial inflammation in mice lacking the interleukin 1 receptor antagonist gene. J Exp Med 2000;191:303-12.
7. Shepherd J, Little MC, Nicklin MJ. Psoriasis-like cutaneous inflammation in mice lacking interleukin-1 receptor antagonist. J Invest Dermatol 2004;122:665-9.
8. Horai R, Nakajima A, Habiro K, Kotani M, Nakae S, Matsuki T, et al. TNF-α is crucial for the development of autoimmune arthritis in IL-1 receptor antagonist‐deficient mice. Journal of Clinical Investigation 2004;114:1603-11.
9. Matsuki T, Isoda K, Horai R, Nakajima A, Aizawa Y, Suzuki K, et al. Involvement of tumor necrosis factor-alpha in the development of T cell-dependent aortitis in interleukin-1 receptor antagonist-deficient mice. Circulation 2005;112:1323-31.
10. Washino T, Moroda M, Iwakura Y, Aosai F. Toxoplasma gondii infection inhibits Th17-mediated spontaneous development of arthritis in interleukin-1 receptor antagonist-deficient mice. Infect Immun

2012;80:1437-44.

11. Fujii H, Baba T, Ishida Y, Kondo T, Yamagishi M, Kawano M, et al. Ablation of the Ccr2 gene exacerbates polyarthritis in interleukin-1 receptor antagonist-deficient mice. Arthritis Rheum 2011;63:96-106.
12. Hirota H, Yoshida K, Kishimoto T, Taga T. Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. Proc Natl Acad Sci U S A 1995;92:4862-6.
13. Van den Herik-Oudijk IE, Ter Bekke MW, Tempelman MJ, Capel PJ, Van de Winkel JG. Functional differences between two Fc receptor ITAM signaling motifs. Blood 1995;86:3302-7.
14. Rogers KA, Scinicariello F, Attanasio R. IgG Fc receptor III homologues in nonhuman primate species: genetic characterization and ligand interactions. J Immunol 2006;177:3848-56.
15. Suzuki H, Yasukawa K, Saito T, Anzai M, Goitsuka R, Hasegawa A, et al. Anti-murine IL-6 receptor antibody inhibits IL-6 effects in vivo. Immunol Lett 1991;30:17-21.
16. Saito T, Yasukawa K, Suzuki H, Futatsugi K, Fukunaga T, Yokomizo C, et al. Preparation of soluble murine IL-6 receptor and anti-murine IL-6 receptor antibodies. J Immunol 1991;147:168-73.
17. Arima H, Hanada M, Hayasaka T, Masaki N, Omura T, Xu D, et al. Blockade of IL-6 signaling by MR16-1 inhibits reduction of docosahexaenoic acid-containing phosphatidylcholine levels in a mouse model of spinal cord injury. Neuroscience 2014;269:1-10.
18. Birner P, Heider S, Petzelbauer P, Wolf P, Kornauth C, Kuroll M, et al. Interleukin-6 receptor alpha blockade improves skin lesions in a murine model of systemic lupus erythematosus. Exp Dermatol 2016;25:305-10.
19. Iwanami K, Matsumoto I, Tanaka-Watanabe Y, Inoue A, Mihara M, Ohsugi Y, et al. Crucial role of the interleukin-6/interleukin-17 cytokine axis in the induction of arthritis by glucose-6-phosphate isomerase. Arthritis Rheum 2008;58:754-63.
20. Ohtsuji M, Lin Q, Nishikawa K, Ohtsuji N, Okazaki H, Tsurui H, et al. IL-6 signal blockade ameliorates the enhanced osteoclastogenesis and the associated joint destruction in a novel FcgammaRIIB-deficient rheumatoid arthritis mouse model. Mod Rheumatol 2015;25:270-7.
21. Suzuki M, Yoshida H, Hashizume M, Tanaka K, Matsumoto Y. Blockade of

interleukin-6 receptor enhances the anti-arthritic effect of glucocorticoids without decreasing bone mineral density in mice with collagen-induced arthritis. Clin Exp Immunol 2015;182:154-61.

22. Yoshida H, Suzuki M, Tanaka K, Takeda S, Yogo K, Matsumoto Y. Anti-interleukin-6 receptor antibody prevents loss of bone structure and bone strength in collagen-induced arthritis mice. Scand J Rheumatol 2018;47:384-91.
23. Latourte A, Cherifi C, Maillet J, Ea HK, Bouaziz W, Funck-Brentano T, et al. Systemic inhibition of IL-6/Stat3 signalling protects against experimental osteoarthritis. Ann Rheum Dis 2017;76:748-55.
24. Okuyama M, Uchida HA, Hada Y, Kakio Y, Otaka N, Umebayashi R, et al. Exogenous Vasohibin-2 Exacerbates Angiotensin II-Induced Ascending Aortic Dilation in Mice. Circulation Reports 2019;1:155-61.
25. Umebayashi R, Uchida HA, Kakio Y, Subramanian V, Daugherty A, Wada J. Cilostazol Attenuates Angiotensin II-Induced Abdominal Aortic Aneurysms but Not Atherosclerosis in Apolipoprotein E-Deficient Mice. Arterioscler Thromb Vasc Biol 2018;38:903-12.
26. Akagi T, Mukai T, Mito T, Kawahara K, Tsuji S, Fujita S, et al. Effect of Angiotensin II on Bone Erosion and Systemic Bone Loss in Mice with Tumor Necrosis Factor-Mediated Arthritis. Int J Mol Sci 2020;21.
27. Kojima F, Kapoor M, Yang L, Fleishaker EL, Ward MR, Monrad SU, et al. Defective generation of a humoral immune response is associated with a reduced incidence and severity of collagen-induced arthritis in microsomal prostaglandin E synthase-1 null mice. Journal of Immunology 2008;180:8361-8.
28. Mukai T, Gallant R, Ishida S, Kittaka M, Yoshitaka T, Fox DA, et al. Loss of SH3 domain-binding protein 2 function suppresses bone destruction in tumor necrosis factor-driven and collagen-induced arthritis in mice. Arthritis Rheumatol 2015;67:656-67.
29. Mihara M, Nishimoto N, Yoshizaki K, Suzuki T. Influences of anti-mouse interleukin-6 receptor antibody on immune responses in mice. Immunol Lett 2002;84:223-9.
30. Bernad A, Kopf M, Kulbacki R, Weich N, Koehler G, Gutierrez-Ramos JC. Interleukin-6 is required in vivo for the regulation of stem cells and committed progenitors of the hematopoietic system. Immunity 1994;1:725-31.

31. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta 2011;1813:878-88.
32. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. Blood 1994;83:113-8.
33. Diao H, Kohanawa M. Endogenous interleukin-6 plays a crucial protective role in streptococcal toxic shock syndrome via suppression of tumor necrosis factor alpha production. Infect Immun 2005;73:3745-8.
34. Tagawa Y, Matthys P, Heremans H, Dillen C, Zaman Z, Iwakura Y, et al. Bimodal role of endogenous interleukin-6 in concanavalin A-induced hepatitis in mice. J Leukoc Biol 2000;67:90-6.
35. Yimin, Kohanawa M. A regulatory effect of the balance between TNF-alpha and IL-6 in the granulomatous and inflammatory response to Rhodococcus aurantiacus infection in mice. J Immunol 2006;177:642-50.
36. Noris M, Daina E, Gamba S, Bonazzola S, Remuzzi G. Interleukin-6 and RANTES in Takayasu arteritis: a guide for therapeutic decisions? Circulation 1999;100:55-60.
37. Park MC, Lee SW, Park YB, Lee SK. Serum cytokine profiles and their correlations with disease activity in Takayasu's arteritis. Rheumatology (Oxford) 2006;45:545-8.
38. Alibaz-Oner F, Yentur SP, Saruhan-Direskeneli G, Direskeneli H. Serum cytokine profiles in Takayasu's arteritis: search for biomarkers. Clin Exp Rheumatol 2015;33:S-32-5.
39. Saadoun D, Garrido M, Comarmond C, Desbois AC, Domont F, Savey L, et al. Th1 and Th17 cytokines drive inflammation in Takayasu arteritis. Arthritis Rheumatol 2015;67:1353-60.
40. Kong X, Sun Y, Ma L, Chen H, Wei L, Wu W, et al. The critical role of IL-6 in the pathogenesis of Takayasu arteritis. Clin Exp Rheumatol 2016;34:S21-7.
41. Nakaoka Y, Isobe M, Takei S, Tanaka Y, Ishii T, Yokota S, et al. Efficacy and safety of tocilizumab in patients with refractory Takayasu arteritis: results from a randomised, double-blind, placebo-controlled, phase 3 trial in Japan (the TAKT study). Ann Rheum Dis 2018;77:348-54.
42. Stone JH, Tuckwell K, Dimonaco S, Klearman M, Aringer M, Blockmans D,

et al. Trial of Tocilizumab in Giant-Cell Arteritis. N Engl J Med 2017;377:317-28.

43. Frey N, Katus HA, Olson EN, Hill JA. Hypertrophy of the heart: a new therapeutic target? Circulation 2004;109:1580-9.
44. Kumar S, Wang G, Zheng N, Cheng W, Ouyang K, Lin H, et al. HIMF (Hypoxia-Induced Mitogenic Factor)-IL (Interleukin)-6 Signaling Mediates Cardiomyocyte-Fibroblast Crosstalk to Promote Cardiac Hypertrophy and Fibrosis. Hypertension 2019;73:1058-70.
45. Meier H, Bullinger J, Marx G, Deten A, Horn LC, Rassler B, et al. Crucial role of interleukin-6 in the development of norepinephrine-induced left ventricular remodeling in mice. Cell Physiol Biochem 2009;23:327-34.
46. Fischer P, Hilfiker-Kleiner D. Survival pathways in hypertrophy and heart failure: the gp130-STAT axis. Basic Res Cardiol 2007;102:393-411.
47. Zhao L, Cheng G, Jin R, Afzal MR, Samanta A, Xuan YT, et al. Deletion of Interleukin-6 Attenuates Pressure Overload-Induced Left Ventricular Hypertrophy and Dysfunction. Circ Res 2016;118:1918-29.
48. Bian Z, Cai J, Shen DF, Chen L, Yan L, Tang Q, et al. Cellular repressor of E1A-stimulated genes attenuates cardiac hypertrophy and fibrosis. J Cell Mol Med 2009;13:1302-13.
49. Qi HP, Wang Y, Zhang QH, Guo J, Li L, Cao YG, et al. Activation of peroxisome proliferator-activated receptor gamma (PPARgamma) through NF-kappaB/Brg1 and TGF-beta1 pathways attenuates cardiac remodeling in pressure-overloaded rat hearts. Cell Physiol Biochem 2015;35:899-912.
50. Wu QQ, Yuan Y, Jiang XH, Xiao Y, Yang Z, Ma ZG, et al. OX40 regulates pressure overload-induced cardiac hypertrophy and remodelling via CD4+ T-cells. Clin Sci (Lond) 2016;130:2061-71.
51. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, et al. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. Proc Natl Acad Sci U S A 1995;92:1142-6.
52. Narazaki M, Yasukawa K, Saito T, Ohsugi Y, Fukui H, Koishihara Y, et al. Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130. Blood 1993;82:1120-6.
53. Wang X, Liu Y, Xiao L, Li L, Zhao X, Yang L, et al. Hyperoside Protects Against Pressure Overload-Induced Cardiac Remodeling via the AKT

Signaling Pathway. Cell Physiol Biochem 2018;51:827-41.

54. Uchiyama Y, Yorozu K, Hashizume M, Moriya Y, Mihara M. Tocilizumab, a humanized anti-interleukin-6 receptor antibody, ameliorates joint swelling in established monkey collagen-induced arthritis. Biol Pharm Bull 2008;31:1159-63.
55. Izumiyama T, Mori Y, Mori S, Mori N, Kodama T, Itoi E. The effect of anti-IL-6 receptor antibody for the treatment of McH-lpr/lpr-RA1 mice that spontaneously developed destructive arthritis and enthesitis. BMC Musculoskelet Disord 2019;20:286.
56. Scott LJ. Tocilizumab: A Review in Rheumatoid Arthritis. Drugs 2017;77:1865-79.
57. Colmegna I, Ohata BR, Menard HA. Current understanding of rheumatoid arthritis therapy. Clin Pharmacol Ther 2012;91:607-20.
58. Alonzi T, Fattori E, Lazzaro D, Costa P, Probert L, Kollias G, et al. Interleukin 6 is required for the development of collagen-induced arthritis. J Exp Med 1998;187:461-8.
59. Ohshima S, Saeki Y, Mima T, Sasai M, Nishioka K, Nomura S, et al. Interleukin 6 plays a key role in the development of antigen-induced arthritis. Proc Natl Acad Sci U S A 1998;95:8222-6.
60. Atsumi T, Ishihara K, Kamimura D, Ikushima H, Ohtani T, Hirota S, et al. A point mutation of Tyr-759 in interleukin 6 family cytokine receptor subunit gp130 causes autoimmune arthritis. J Exp Med 2002;196:979-90.
61. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143-50.
62. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol 2010;10:301-16.
63. Silman AJ. Available therapeutic options following failure of a first anti-TNF agent. Nat Clin Pract Rheumatol 2009;5:115.
64. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nat Rev Immunol 2007;7:429-42.
65. Kopf M, Bachmann MF, Marsland BJ. Averting inflammation by targeting the cytokine environment. Nat Rev Drug Discov 2010;9:703-18.
66. Ogura H, Murakami M, Okuyama Y, Tsuruoka M, Kitabayashi C,

Kanamoto M, et al. Interleukin-17 promotes autoimmunity by triggering a positive-feedback loop via interleukin-6 induction. Immunity 2008;29:628-36.

67. Nishimoto N, Kishimoto T. Inhibition of IL-6 for the treatment of inflammatory diseases. Curr Opin Pharmacol 2004;4:386-91.
68. Aksentijevich I, Masters SL, Ferguson PJ, Dancey P, Frenkel J, van Royen-Kerkhoff A, et al. An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. N Engl J Med 2009;360:2426-37.
69. Reddy S, Jia S, Geoffrey R, Lorier R, Suchi M, Broeckel U, et al. An autoinflammatory disease due to homozygous deletion of the IL1RN locus. N Engl J Med 2009;360:2438-44.
70. Jesus AA, Osman M, Silva CA, Kim PW, Pham TH, Gadina M, et al. A novel mutation of IL1RN in the deficiency of interleukin-1 receptor antagonist syndrome: description of two unrelated cases from Brazil. Arthritis Rheum 2011;63:4007-17.
71. Brau-Javier CN, Gonzales-Chavez J, Toro JR. Chronic cutaneous pustulosis due to a 175-kb deletion on chromosome 2q13: excellent response to anakinra. Arch Dermatol 2012;148:301-4.
72. Stenerson M, Dufendach K, Aksentijevich I, Brady J, Austin J, Reed AM. The first reported case of compound heterozygous IL1RN mutations causing deficiency of the interleukin-1 receptor antagonist. Arthritis Rheum 2011;63:4018-22.

Table 1. Characteristics of study mice

Genotype	**wild**	**KO**	**KO**	**KO**
Treatment	**IgG**	**IgG**	**MR-16 low**	**MR-16 high**
N (M/F)	19 (9/10)	22 (10/12)	14 (6/8)	20 (8/12)
SBP, mmHg	101 ± 12	109 ± 4	103 ± 13	106 ± 18
HR, bpm	533 ± 73	575 ± 75	569 ± 58	554 ± 67
BW, g	22.5 ± 3.3	19.3 ± 2.4*	19.1 ± 2.5*	18.8 ± 2.7*

N: number, M: male, F: female, SBP: systolic blood pressure, HR: heart rate,

BW: body weight, mean ± SD, *P < 0.05 vs wild/IgG (One-way ANOVA on Rank)

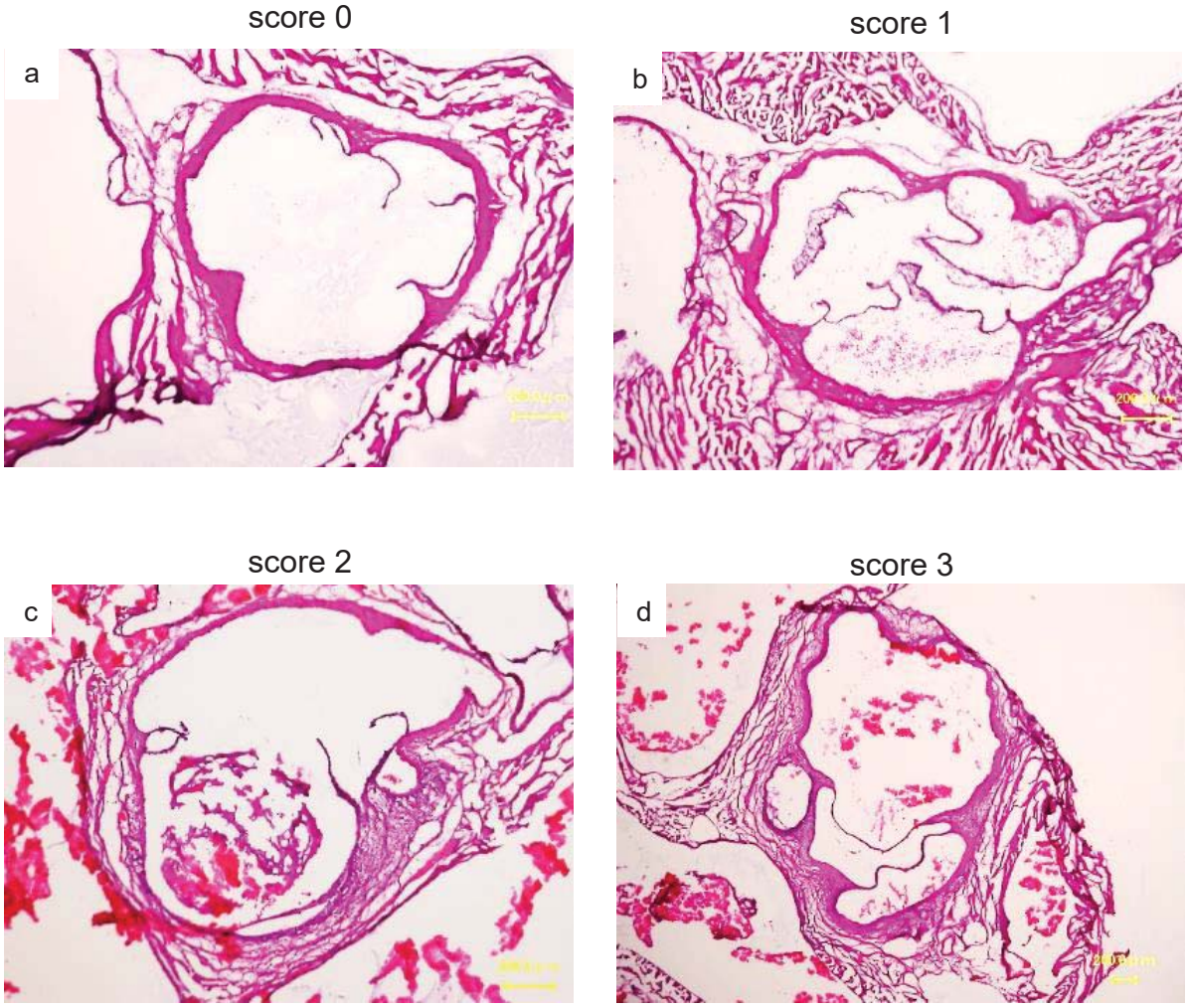
Table 2. Heart weight data of study mice

Genotype	**wild**	**KO**	**KO**	**KO**
Treatment	**IgG**	**IgG**	**MR-16 low**	**MR-16 high**
N (M/F)	19 (9/10)	22 (10/12)	14 (6/8)	20 (8/12)
HW, mg	147.3 ± 34.0	126.4 ± 27.3*	133.4 ± 15.3	124.5 ± 16.2*
BW, g	22.5 ± 3.3	19.3 ± 2.4*	19.1 ± 2.5*	18.8 ± 2.7*
HW/BW, mg/g	6.5 ± 1.2	6.6 ± 1.2	7.1 ± 0.9	6.7 ± 0.9

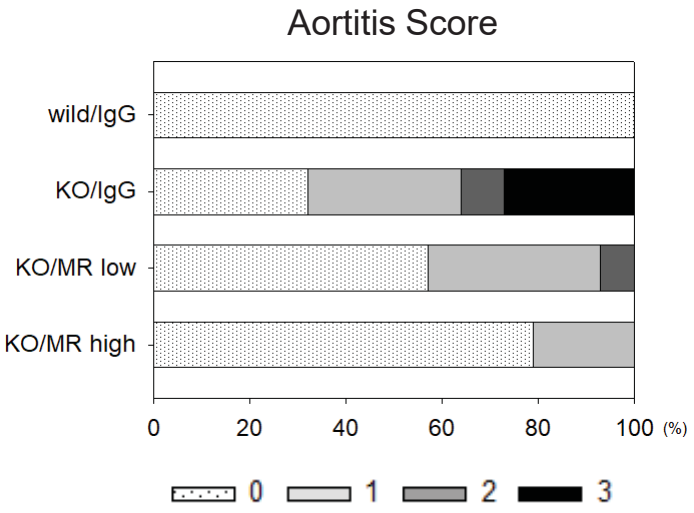
HW: heart weight, BW: body weight, mean ± SD, *$P < 0.05$ vs wild/IgG (One-way ANOVA on Rank)

Figure 1

A



B



C

Severity of Aortitis

	normal-mild (score 0 and 1)	Moderate-severe (score 2 and 3)
KO/IgG	14	8 (2)
KO/MR high	19	0

P = 0.00414 (Fisher Exact Test)

(n) : death

Figure 2

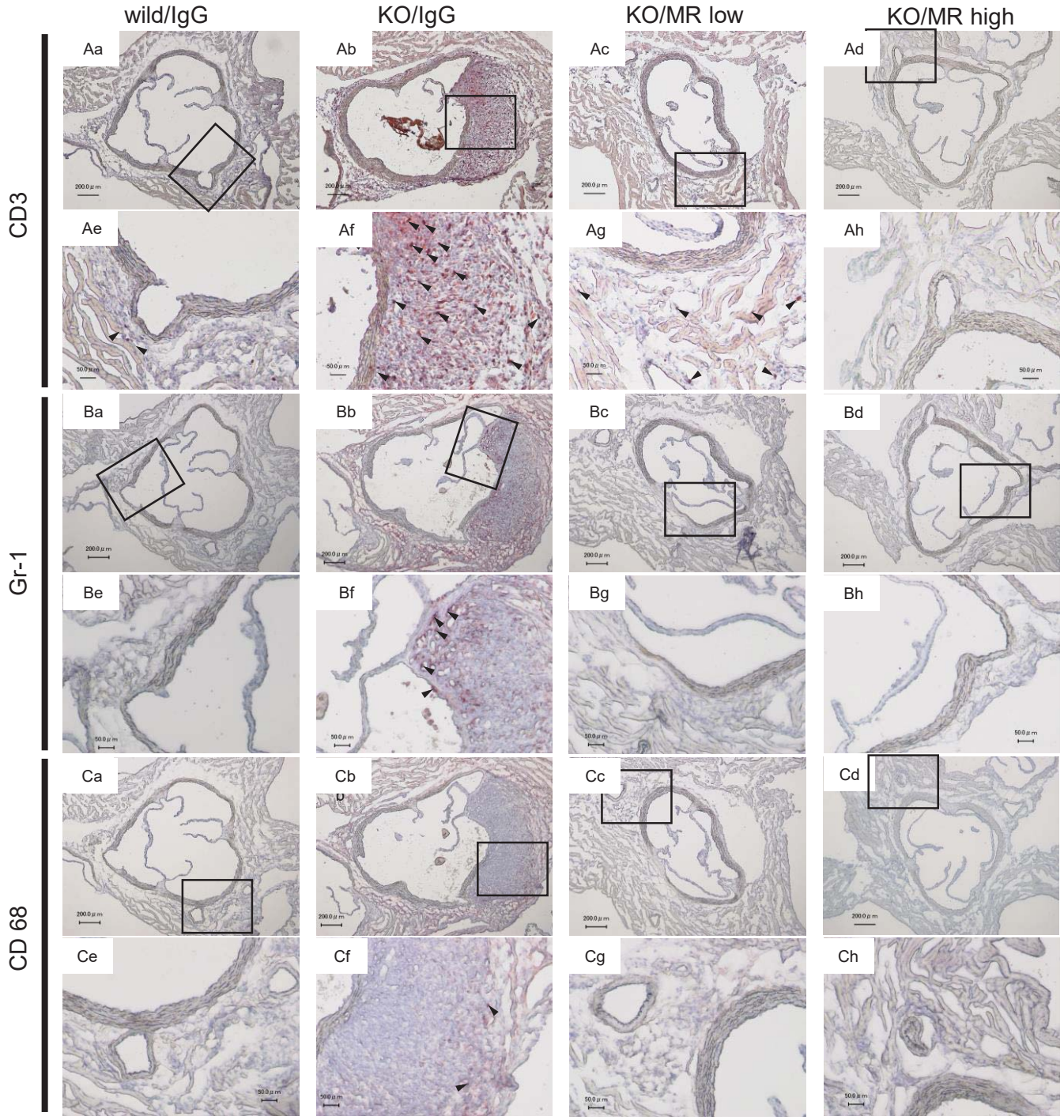

wild/IgG
KO/IgG
KO/MR low
KO/MR high
CD3
Gr-1
CD 68
Aa
Ab
Ac
Ad
Ae
Af
Ag
Ah
Ba
Bb
Bc
Bd
Be
Bf
Bg
Bh
Ca
Cb
Cc
Cd
Ce
Cf
Cg
Ch

Figure 3

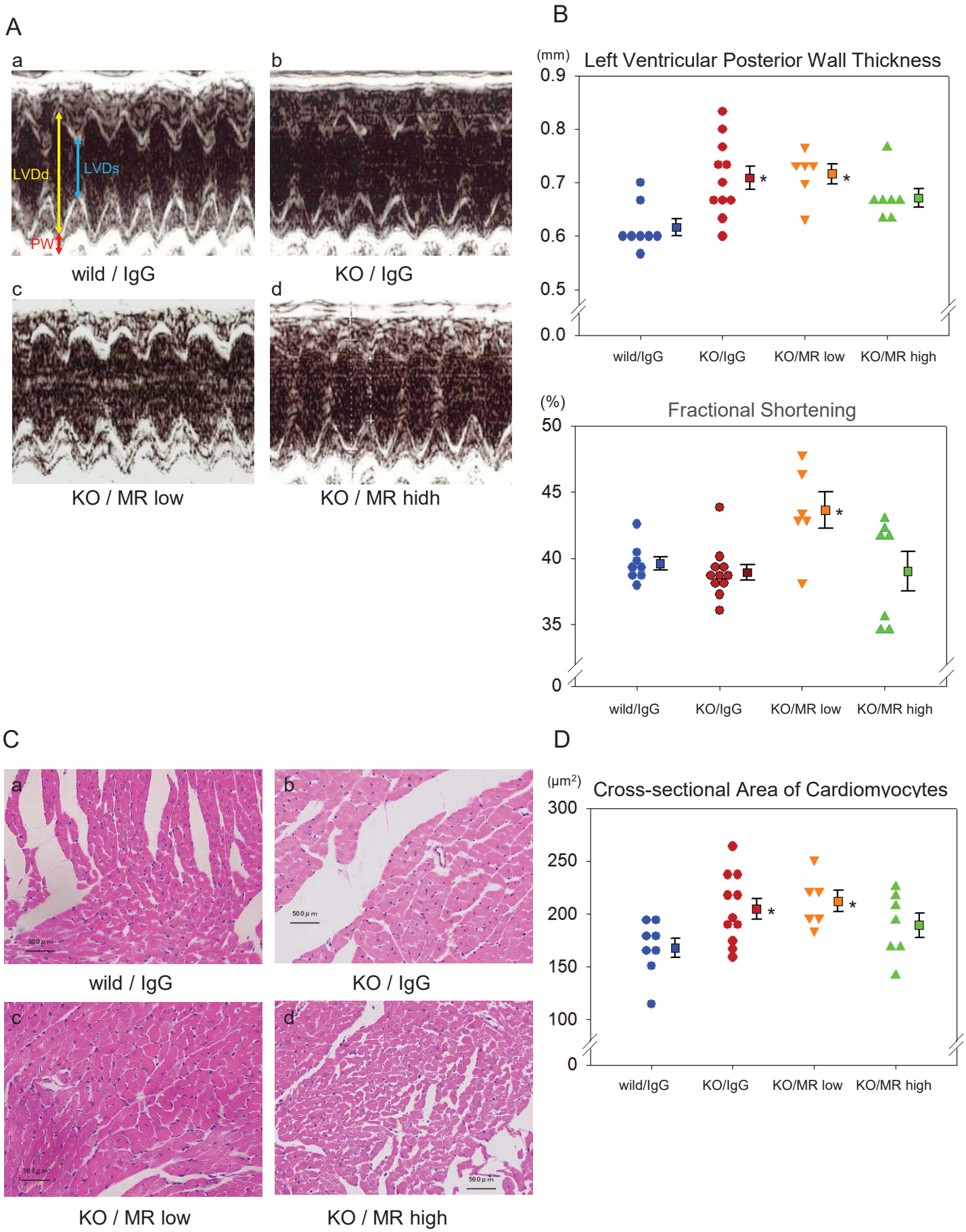

A
a
LVDd
LVDs
PW
wild / IgG
b
KO / IgG
c
KO / MR low
d
KO / MR hidh
B
(mm)
Left Ventricular Posterior Wall Thickness
0.9
0.8
0.7
0.6
0.5
0.0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(%)
Fractional Shortening
50
45
40
35
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
C
a
wild / IgG
b
KO / IgG
c
KO / MR low
d
KO / MR high
D
(μm²)
Cross-sectional Area of Cardiomyocytes
300
250
200
150
100
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high

Figure 4

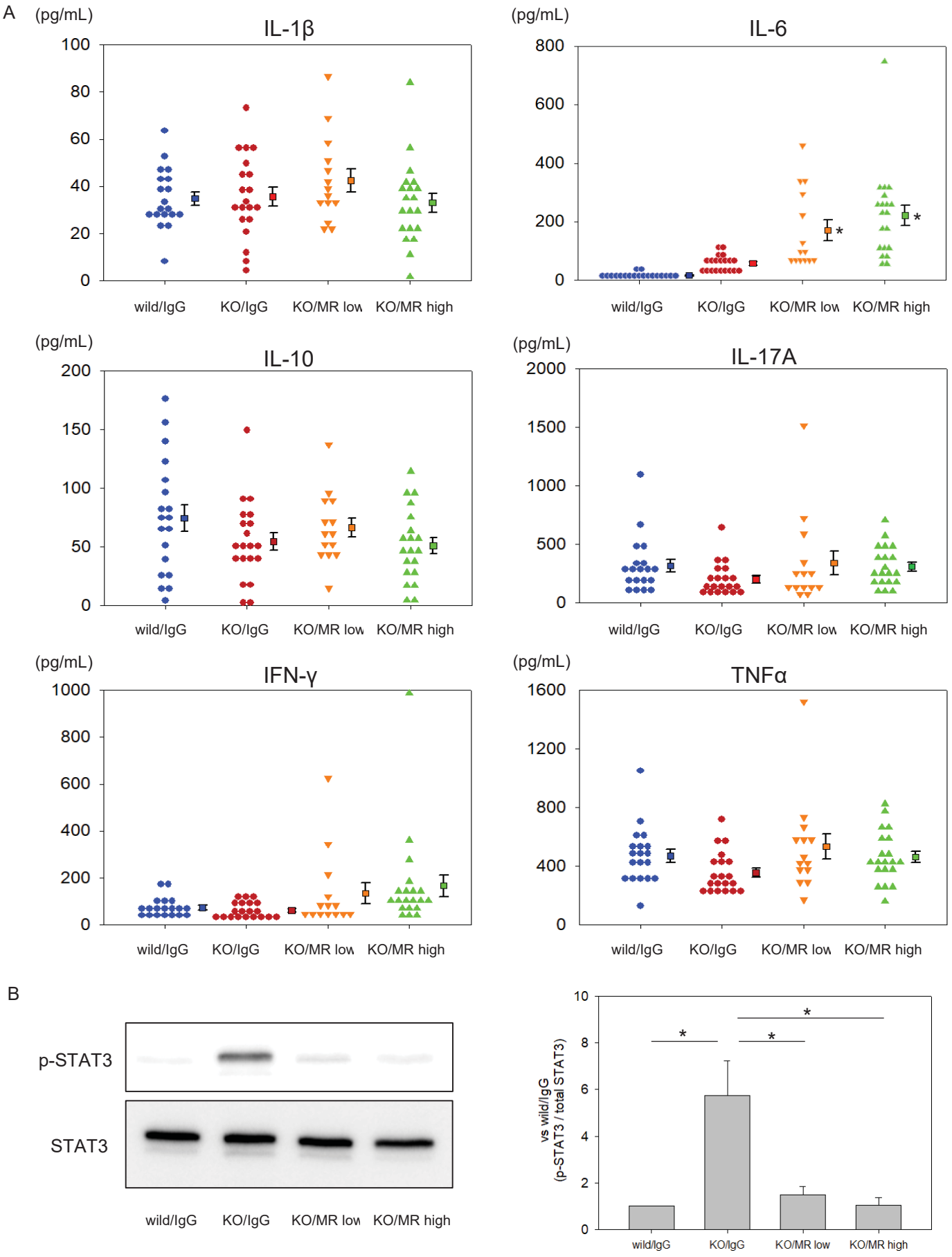

A
(pg/mL)
IL-1β
100
80
60
40
20
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(pg/mL)
IL-6
800
600
400
200
0
*
*
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(pg/mL)
IL-10
200
150
100
50
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(pg/mL)
IL-17A
2000
1500
1000
500
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(pg/mL)
IFN-γ
1000
800
600
400
200
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
(pg/mL)
TNFα
1600
1200
800
400
0
wild/IgG
KO/IgG
KO/MR low
KO/MR high
B
p-STAT3
STAT3
wild/IgG
KO/IgG
KO/MR low
KO/MR high
vs wild/IgG
(p-STAT3 / total STAT3)
10
8
6
4
2
0
*
*
*
wild/IgG
KO/IgG
KO/MR low
KO/MR high

Figure 5

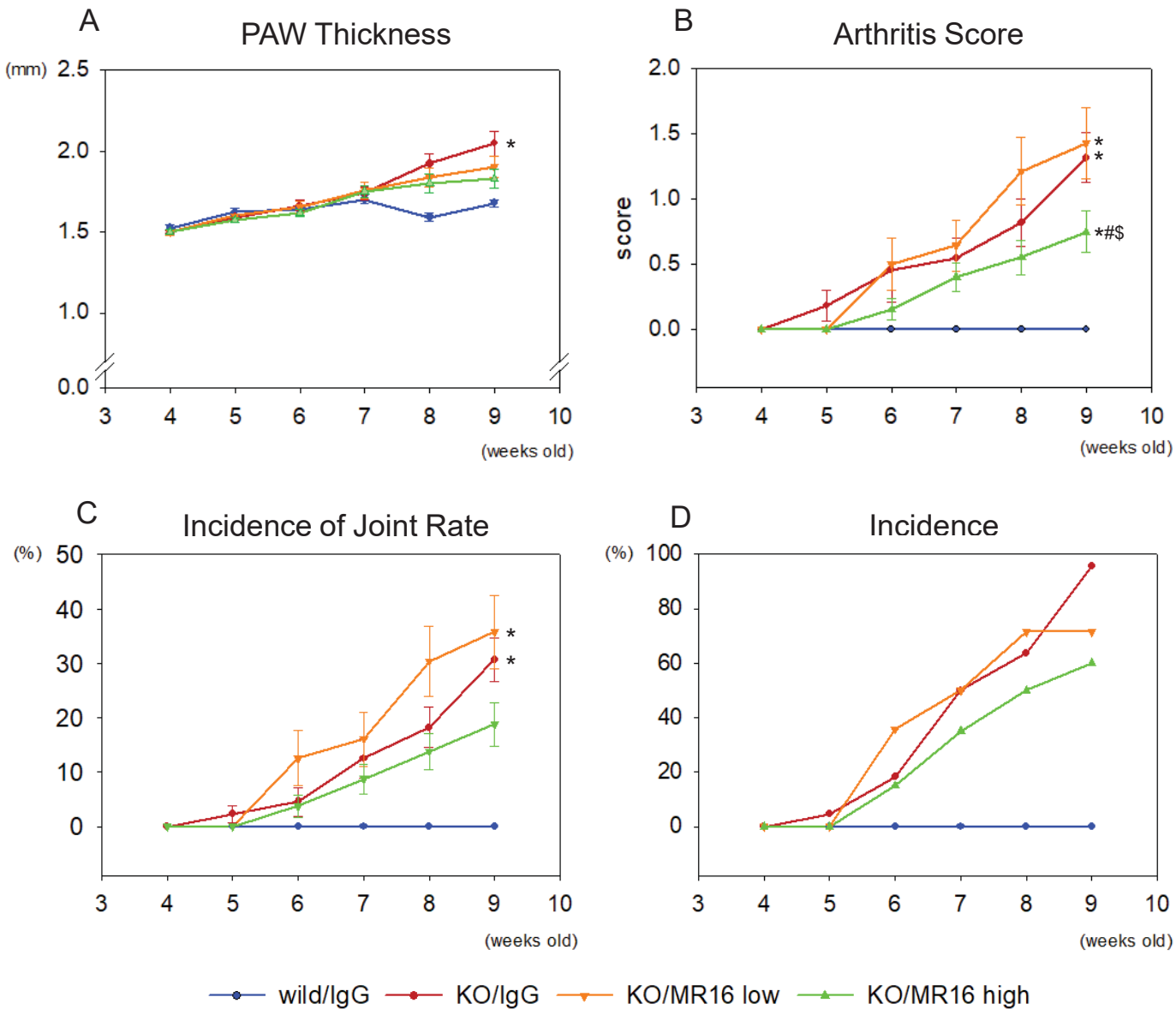
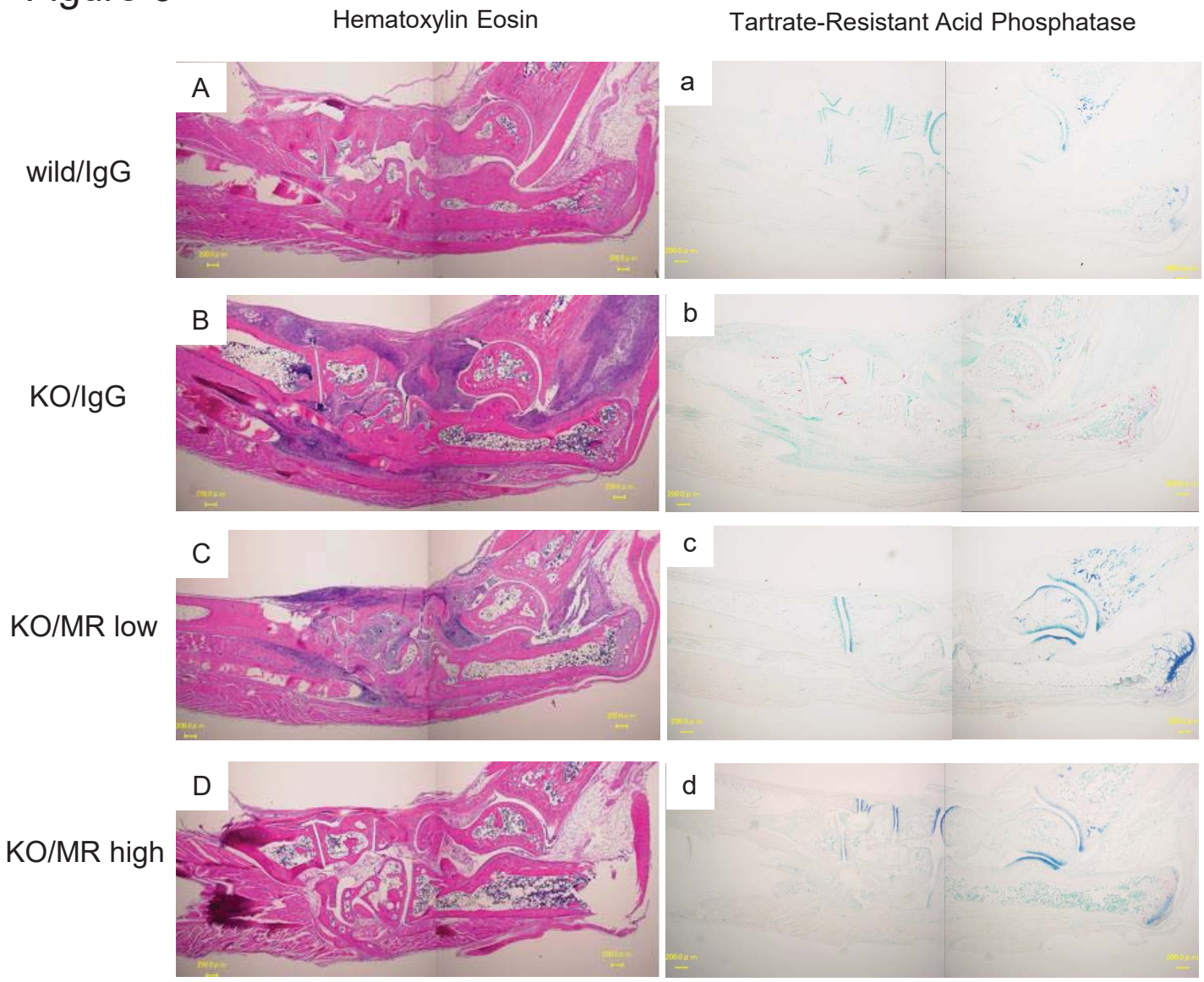
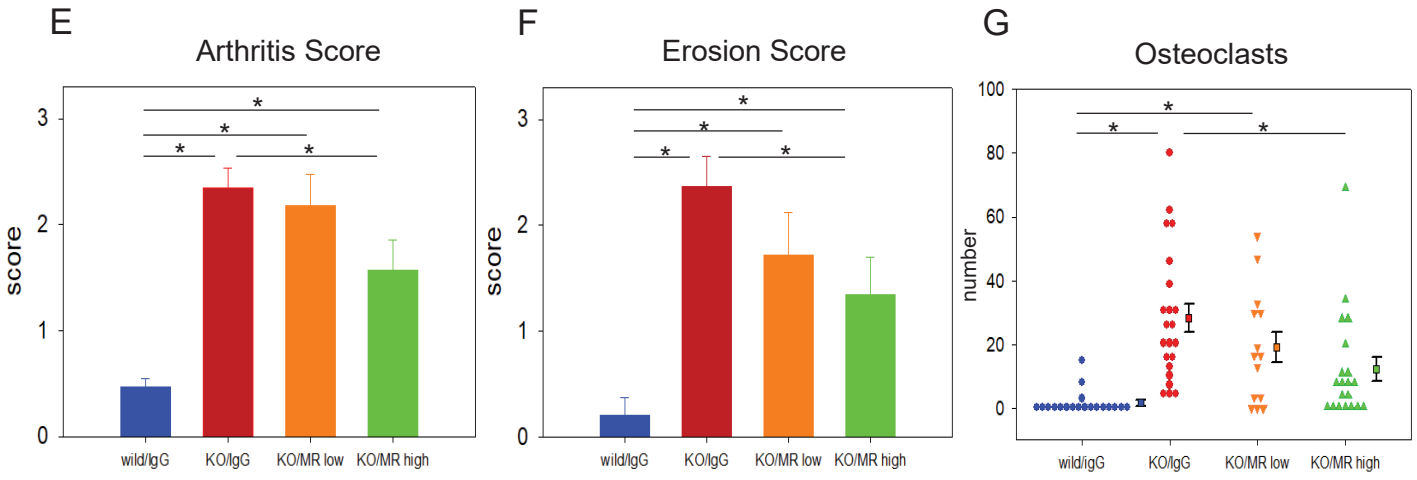

A
PAW Thickness
(mm)
2.5
2.0
1.5
1.0
0.0
3
4
5
6
7
8
9
10
(weeks old)
*
B
Arthritis Score
score
2.0
1.5
1.0
0.5
0.0
*
*
*#$
(weeks old)
C
Incidence of Joint Rate
(%)
50
40
30
20
10
0
*
*
(weeks old)
D
Incidence
(%)
100
80
60
40
20
0
(weeks old)
wild/IgG
KO/IgG
KO/MR16 low
KO/MR16 high

Figure 6


Hematoxylin Eosin
Tartrate-Resistant Acid Phosphatase
A
a
wild/IgG
B
b
KO/IgG
C
c
KO/MR low
D
d
KO/MR high


E
Arthritis Score
score
wild/IgG
KO/IgG
KO/MR low
KO/MR high
F
Erosion Score
score
wild/IgG
KO/IgG
KO/MR low
KO/MR high
G
Osteoclasts
number
wild/igG
KO/IgG
KO/MR low
KO/MR high

Supplemental Material

Inhibition of Interleukin-6 Signaling Attenuates Aortitis, Left Ventricular Hypertrophy and Arthritis in Interleukin-1 Receptor Antagonist Deficient Mice

Yoshiko Hada[1,2], Haruhito A. Uchida[1,2], Tomoyuki Mukai[3], Fumiaki Kojima[4], Masashi Yoshida[2,5], Hidemi Takeuchi[1], Yuki Kakio[1], Nozomu Otaka[1,6], Yoshitaka Morita[3], Jun Wada[1]

1. Department of Nephrology, Rheumatology, Endocrinology and Metabolism, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
2. Department of Chronic Kidney Disease and Cardiovascular Disease, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
3. Department of Rheumatology, Kawasaki Medical School, Kurashiki, Japan.
4. Department of Pharmacology, Kitasato University School of Allied Health Sciences, Sagamihara, Japan
5. Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.
6. Department of Human Resource Development of Dialysis Therapy for Kidney Disease, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Science, Okayama, Japan.

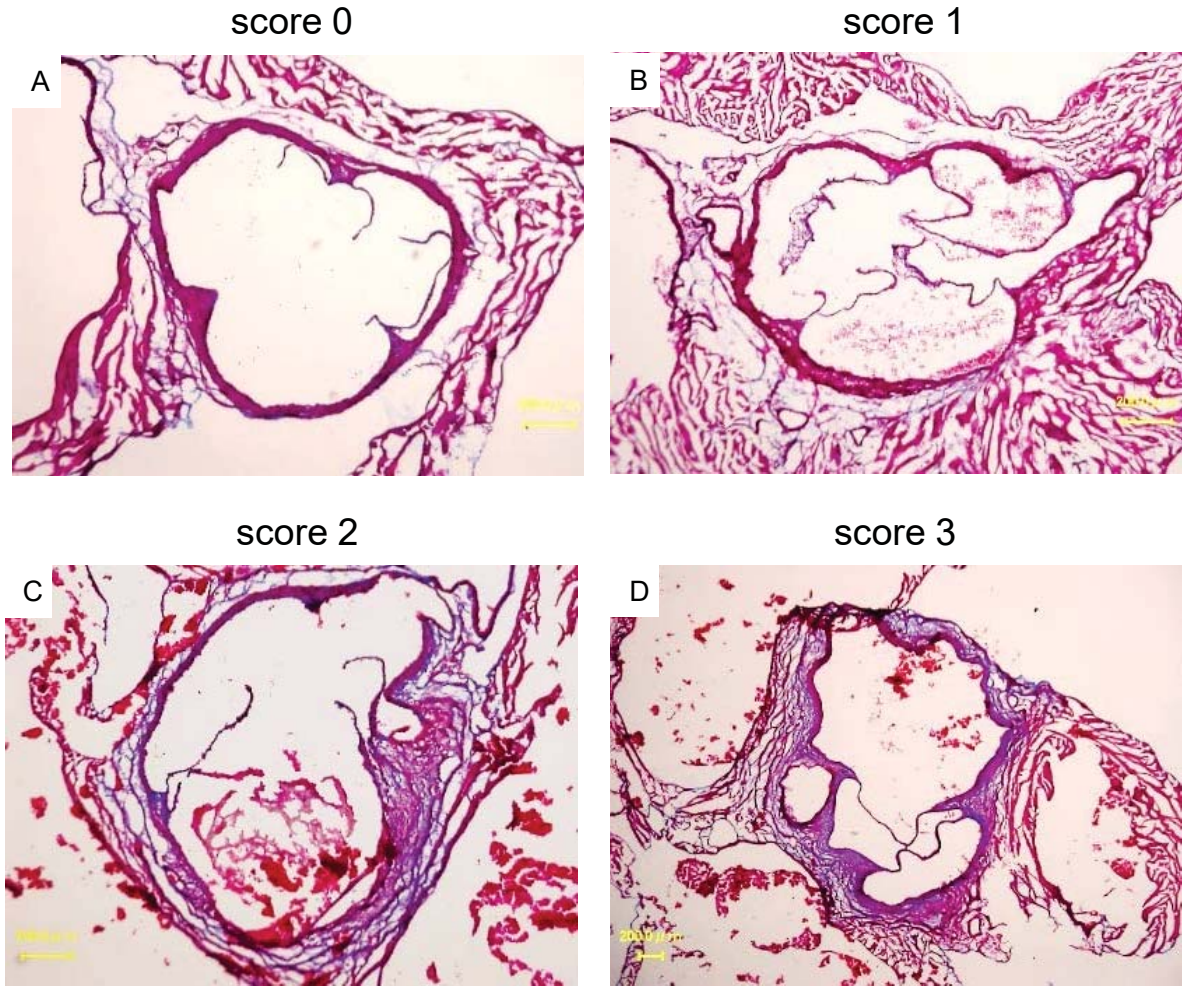
Running Title:
IL-6 Inhibition on aortitis, LVH, and arthritis (47 characters)

Corresponding author:
Haruhito A. Uchida, M.D., Ph.D.
Department of Chronic Kidney Disease and Cardiovascular Disease,
Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,
2-5-1 Shikata-cho Kita-ku, Okayama, 700-8558, Japan
Phone: (+81) 86-235-7235 Fax: (+81) 86-222-5214

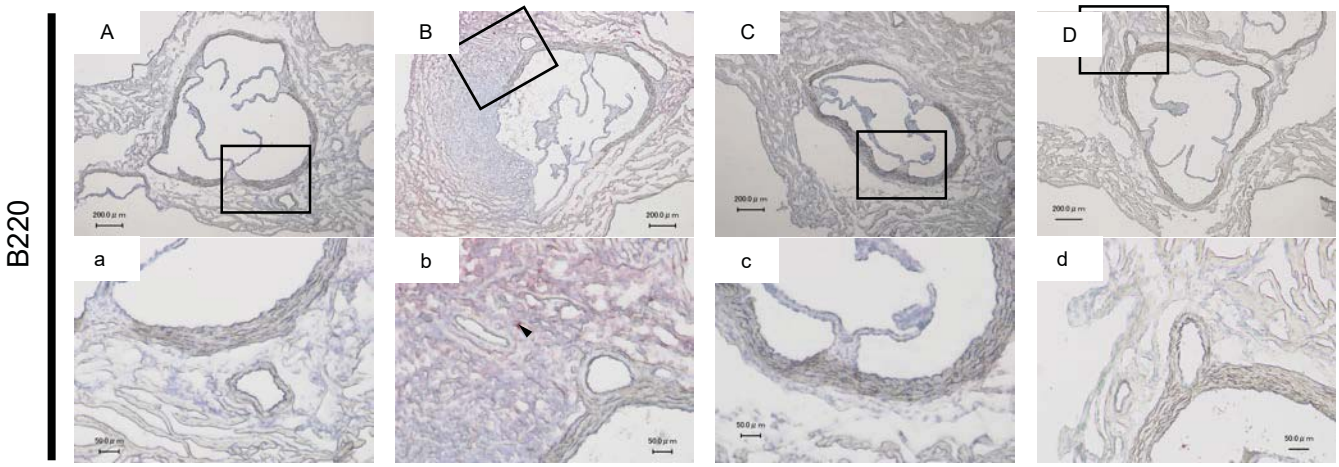
E-mail: hauchida@okayama-u.ac.jp

Key Word: MR16-1, interleukin-1 receptor antagonist, aortitis, arthritis, LV hypertrophy

Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure Legend

Supplemental Figure 1. Score for inflammation of arteries around the aortic sinus in IL-1RA KO mice.
A, The severity of aortitis was graded on a scale of 0 to 3 by the degree of inflammation near the aortic valve from 8-week-old mice. Representative photos of masson's trichrome stains (A - D) of the aortic sinus are shown in each score. The scale bar indicates 200 μm.

Supplemental Figure 2. Assessment of B220 positive cells in aortitis lesions. Representative photos of immunostainings are shown, the wild/IgG mice (A and a), the KO/IgG mice (B and b), the KO/MR low mice (C and c), and the KO/MR high mice (D and d) were sacrificed at 9-weeks-old, and aortic cryosections were obtained. These sections were stained with mAbs against B220 (A – D, a - d). The scale bar indicates 200 μm (A - D) and 50 μm (a - d).