Treatment with CCR9 antagonist did not accompany critical infections as an adverse effect in a large phase II study for Crohn's disease [18,19]. Although the reason is not clear, it might be also the case for treating RA.

## Conclusions

In this manuscript, we showed that CCL25 and CCR9 were expressed in the RA synovial tissue. Stimulation with CCL25 enhanced production of inflammatory mediators from monocytes and RA FLS. Moreover, inhibition of CCR9 reduced arthritis and inflammatory cell migration in mice. Therefore, the interaction between CCL25 and CCR9 may play important roles in cell infiltration into the RA synovial tissues and inflammatory mediator production. CCR9 antagonist may become a novel, safe and effective treatment for RA.

### Abbreviations

ANOVA: analysis of variance; AUC: area under the curve; CCL: chemokine (C-C motif) ligand; CCR: CC chemokine receptor; CIA: collagen-induced arthritis; CII: type II collagen; CMTMR: 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine; DC: dendritic cell, DC-LAMP, dendritic cell lysosome-associated membrane glycoprotein; ELISA: enzyme-linked immunosorbent assays; FCS: fetal calf serum; FI: fluorescence intensity; FLS: fibroblast-like synoviocytes; IL: interleukin; KO: knockout; mAb: monoclonal antibody; MFI: mean fluorescence intensity; MMP: metalloproteinase; OA: osteoarthritis; RA: rheumatoid arthritis; SEM: standard error of the mean; TNF: tumor necrosis factor; WT: wild-type.

### Competing interests

Drs. Walters, Charvat, Penfold, Jaen and Schall own stock or stock options in ChemoCentryx, Inc. The other authors declare that they have no competing interests.

### Authors' contributions

WY participated in the design of the study, carried out the experiments and statistical analysis, and drafted the manuscript. KK, AT, SF, CM, YM and KW assisted in carrying out the experiments and in manuscript preparation. MW, TC, MP, JJ and TS provided the CCR9 antagonist, participated in study design, and assisted in manuscript preparation. PL, NN and TK provided CCR9-deficient mice and assisted in manuscript preparation. HK, MH, NM and TN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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# Cannabinoid receptor 2 as a potential therapeutic target in rheumatoid arthritis

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## Abstract

**Background:** Some of cannabinoids, which are chemical compounds contained in marijuana, are immunosuppressive. One of the receptors, CB receptor 1 ($CB_1$), is expressed predominantly by the cells in the central nervous system, whereas CB receptor 2 ($CB_2$) is expressed primarily by immune cells. Theoretically, selective $CB_2$ agonists should be devoid of psychoactive effects. In this study, we investigated therapeutic effects of a selective $CB_2$ agonist on arthritis.

**Methods:** The expression of $CB_2$ was analyzed with immunohistochemistry and Western blotting. Interleukin (IL)-6, matrix metalloproteinase-3 (MMP-3), and chemokine (C-C motif) ligand 2 (CCL2) were quantified with enzyme-linked immunosorbent assays (ELISA). Osteoclastogenesis was assessed with tartrate-resistant acid phosphatase staining and the resorption of coated-calcium phosphate. Effect of JWH133, a selective $CB_2$ agonist, on murine collagen type II (CII)-induced arthritis (CIA) was evaluated with arthritis score, and histological and radiographic changes. IFN-γ and IL-17 production by CII-stimulated splenocytes and serum anti-CII Ab were analyzed by ELISA.

**Results:** Immunohistochemistry showed that $CB_2$ was expressed more in the synovial tissues from the rheumatoid joints than in those from the osteoarthritis joints. $CB_2$ expression on RA FLS was confirmed with Western blot analysis. JWH133 inhibited IL-6, MMP-3, and CCL2 production from tumor necrosis factor-α-stimulated fibroblast-like synoviocytes (FLS) derived from the rheumatoid joints, and osteoclastogenesis of peripheral blood monocytes. Administration of JWH133 to CIA mice reduced the arthritis score, inflammatory cell infiltration, bone destruction, and anti-CII IgG1 production.

**Conclusion:** The present study suggests that a selective $CB_2$ agonist could be a new therapy for RA that inhibits production of inflammatory mediators from FLS, and osteoclastogenesis.

**Keywords:** Cannabinoid, Cannabinoid receptor 2 ($CB_2$), Rheumatoid arthritis, JWH133, Fibroblast-like synoviocyte, Monocyte

## Background

Rheumatoid arthritis (RA) is a systemic autoimmune disease of unknown etiology. It is associated with chronic inflammation, bone destruction in multiple joints, and various extra-articular manifestations. Unless treated properly, it is generally progressive with functional decline, significant morbidity, premature mortality, and socioeconomic costs [1]. Recently, biological agents, represented by anti-tumor necrosis factor (TNF) monoclonal antibodies (mAb), have been used widely to improve arthritis and to inhibit bone destruction. However, there remain patients who do not respond satisfactorily. While pain control is a significant issue for the patients, disease-modifying antirheumatic drugs (DMARDs) do not have immediate effects for pain relief. The patients have to depend on corticosteroids or non-steroidal anti-inflammatory drugs (NSAIDs).

Cannabinoids are pharmacologically active components of *Cannabis sativa*. The endogenous ligands for cannabinoid receptors represented by anandamide and

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2-arachidonoylglycerol also occur as endocannabinoids. This system regulates various physiological processes such as appetite control, pain perception and immune responses. Cannabinoids transmit signals through cannabinoid (CB) receptors, CB receptor 1 ($CB_1$) and CB receptor 2 ($CB_2$) [2,3]. $CB_1$ is expressed predominantly by the cells in the central nervous system (CNS), whereas $CB_2$ is expressed primarily by immune cells [2,3] and by peripheral nerve terminals [4]. Recently, G-coupled receptor 55 (GPR55) was proved to be a receptor of many endocannabinoids [5,6]. It is expressed ubiquitously in many organ systems, including CNS [7]. Furthermore, endocannabinoids act as agonists of transient receptor potential vanilloid type-1 and type-4 (TRPV-1, -4) [8,9], and nuclear peroxisome proliferator-activated receptors (PPARs) [10]. Because of the potential effects on a wide variety of the receptors in the various organs, cannabinoids have not been accepted as therapeutic agents. Especially, the major concerns are their psychoactive effects such as catalepsy and hypolocomotion. In this regards, selective $CB_2$ agonists should be devoid of psychoactive activities.

Immune cells sensitive to cannabinoids are macrophages, natural killer cells, T cells, and B cells [11]. Some cannabinoids were applied to treat collagen-induced arthritis (CIA), which is a murine model of RA. HU320, a synthetic cannabinoid ameliorated established CIA [12]. Since HU320 has no affinity for $CB_1$ or $CB_2$, its therapeutic effect on CIA may derive from actions on other receptors. Blockade of fatty acid amide hydrolase (FAAH), which is the primary degradative enzyme of anandamide, and acts as an agonist for $CB_1$, $CB_2$ and other receptors, reduced the severity of CIA [13]. However, therapeutic effect of selective $CB_2$ agonists on animal models of RA has not been investigated.

It was reported that local administration of JWH133, which is a selective $CB_2$ agonist with 200-fold selectivity for $CB_2$ over $CB_1$ (Ki values are 3.4 and 677 nM respectively) [14], inhibited pain reaction of mice with carrageenan-injected paws [15]. NSAIDs and corticosteroids have several toxicities especially in long-term treatment or at high doses. In many cases, opioid analgesics would be better choice for avoiding the toxicities. Add-on therapy for RA patients with tramadol, which is a weak opioid analgesic and acetaminophen combination tablet, significantly improved joint pain without severe adverse effects [16]. Thus, selective $CB_2$ agonists would be expected not only to relief pain, but also to suppress arthritis.

In the present study, we investigated the expression of $CB_2$ in the RA synovial tissues, *in vitro* effects of JWH133 on RA fibroblast-like synoviocytes (FLS) and human monocytes, and its *in vivo* therapeutic effects on CIA.

## Methods

### Specimens

Synovial tissue samples were obtained from seven RA patients, who fulfilled the ACR classification criteria [17] and three osteoarthritis (OA) patients undergoing total knee joint replacement. The RA patients were 62 (38-75) years old [median (range)], with a disease duration of 9 (3-15) years and C-reactive protein level of 4.6 (0.3-81) mg/l. Informed consent was obtained from all the patients. All experimental protocols were approved by the Ethics Committee of Tokyo Medical and Dental University.

### Immunohistochemistry

Immunohistochemical analysis was conducted on formalin-fixed paraffin-embedded sections of synovial tissues. The sections were incubated overnight at 4°C with 2 μg/ml rabbit anti-$CB_2$ polyclonal antibody (pAb) (abcam, Cambridge MA) or normal rabbit IgG as a control. Subsequently, the samples were incubated with 2 μg/ml biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) for 30 min at room temperature, and then incubated for 30 min with streptavidin–horseradish peroxidase (Dako, Glostrup, Denmark). Diaminobenzidine (Dako) was used for visualization. The sections were counterstained with hematoxylin. The $CB_2$ staining of three RA and three OA samples was semi-quantitatively evaluated by randomly selected three fields with scored as follows: 0 = none, 1 = focal, and 2 = diffuse. The maximum score was six for each sample.

For immunofluorescence double-staining with CD68, CD4, CD8, CD21 or vimentin, and $CB_2$, the sections were incubated overnight at 4°C with 2 μg/ml rabbit anti-$CB_2$ pAb or normal rabbit IgG together with 1 μg/ml mouse anti-CD68 mAb (KP1; Dako), 1 μg/ml mouse anti-CD4 mAb (RPA-T4; eBioscience, San Diego, CA), 1 μg/ml mouse anti-CD8 mAb (HIT8a; BD Bioscience, San Diego, CA), 1 μg/ml mouse anti-CD21 mAb (1 F8; Dako) or 1 μg/ml mouse anti-vimentin mAb (V9; Dako). Subsequently, the samples were incubated with 2 μg/ml Alexa Fluor 488-conjugated goat anti-mouse IgG1 (Invitrogen, Grand Island, NY) and Alexa Fluor 568-conjugated goat anti-rabbit IgG1 (Invitrogen) for 30 min at room temperature. A nuclear stain was performed with 4', 6-diamidino-2-phenylindole.

### Protein detection in cultured FLS

FLS from the RA synovial tissues was cultured as was reported previously [18]. RA FLS was lysed with radioimmunoprecipitation assay buffer (Millipore, Billerica, MA, USA) for 30 min at 4°C. A total of 20 μg of protein were boiled in the presence of sodium dodecyl sulfate (SDS) sample buffer and separated on a 10% SDS-polyacrylamide gel (ATTO, Tokyo, Japan). Proteins were then electrotransferred onto a polyvinylidene fluoride microporous membrane (Millipore) in a semidry system. The membrane was blocked with Block Ace (Snow Brand Milk Products, Tokyo, Japan) for 1 h at room temperature, and then the immunoblots were incubated overnight with 1 μg/ml rabbit anti-$CB_2$ pAb in Can Get Signal

Immunoreaction Enhancer Solution (Toyobo, Osaka, Japan) at 4°C. After washing, the immunoblots were incubated with 2 μg/ml biotinylated goat anti-rabbit IgG for 30 min at room temperature, and then incubated for 30 min with streptavidin–horseradish peroxidase. ECL Prime detection reagent and the ImageQuant LAS 4000 Mini Biomolecular Imager (both from GE Healthcare) were used to detect the bands.

RA FLS ($1 \times 10^5$ cells/ml) was cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich) + 10% fetal bovine serum (FBS) (Sigma-Aldrich) and stimulated with 5 ng/ml recombinant TNF-α (R&D Systems, Minneapolis, MN) for 24 h in the presence or absence of JWH133 (Tocris bioscience, Ellisville, MO) [14]. The concentrations of Interleukin (IL)-6, metalloproteinase-3 (MMP-3) and chemokine (C-C motif) ligand 2 (CCL2) in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) kits (DuoSet; R&D Systems).

### Analysis of osteoclastogenesis

Peripheral blood mononuclear cells from healthy donors were collected using Ficoll-Conray (Imuuno-Biological Laboratories, Gunma, Japan) gradient centrifugation. Positive selection of $CD14^+$ monocytes was performed using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA). The purified peripheral blood $CD14^+$ monocytes ($1 \times 10^6$ cells/well) were incubated in 96-well plates in α-Minimum Essential Medium (Sigma-Aldrich) with 10% FBS, and incubated with 25 ng/ml macrophage colony-stimulated factor (M-CSF) (R&D systems) + 40 ng/ml receptor activator of nuclear factor kappa-B ligand (RANKL) (Peprotech, Rocky Hill, NJ). These cells incubated in the presence or absence of JWH133. The medium was replaced with fresh medium 3 days later, and after incubation for 7 days the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression using a commercial kit (Hokudo, Sapporo, Japan). The number of TRAP-positive multinucleated cells (MNC: more than 3 nuclear) in a randomly selected field examined at ×40 magnification was counted under light microscopy. The $CD14^+$ monocytes were seeded onto plates coated with calcium phosphate thin films (Osteo Assay Plate, Corning, NY, USA) and were incubated with 25 ng/ml M-CSF + 40 ng/ml RANKL for 7 days in the presence or absence of JWH133. The cells were then lysed in bleach solution (6% NaOCl, 5.2% NaCl). The resorption lacunae were examined under light microscopy. The viability of the cells treated with JWH133 (up to 50 μM) was more than 95% relative to the vehicle-treated cells.

### Induction of collagen-induced arthritis (CIA)

Male 8-week-old DBA/1 J mice were purchased from Oriental Yeast (Tokyo, Japan) and were kept in the temperature of 23.5 ± 2 degrees Celsius with 40-50% humidity. Bovine collagen type II (CII; Collagen Research Center, Tokyo, Japan) was dissolved in 0.05 M acetic acid at 4 mg/ml and emulsified in equal volume of complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI). Mice were immunized with 100 μl of the emulsion injected intracutaneously at the base of the tail (day 1). After 21 days (day 22), the same amount of bovine CII emulsified in CFA was injected intracutaneously at the base of the tail as a booster immunization [19].

### Treatment of collagen-induced arthritis (CIA) mice with JWH133

Twelve mice with CIA per group were twice daily injected intraperitoneally with JWH133, 1 mg/kg/day or 4 mg/kg/day in total volume of 200 μl/day of 20% dimethyl sulphoxide or vehicle alone from day 15 to day 35. To determine the therapeutic effects of JWH133, we also treated mice with 4 mg/kg JWH133 from day 28, after the development of arthritis, to day 35 and observed the mice for signs of arthritis. Disease severity for each limb was recorded as follows: 0 = normal, 1 = erythema and swelling of one digit, 2 = erythema and swelling of two digits or erythema and swelling of ankle joint, 3 = erythema and swelling of more than three digits or swelling of two digits and ankle joint, and 4 = erythema and severe swelling of the ankle, foot, and digits with deformity. The clinical arthritis score was defined as the sum of the scores for all 4 paws of each mouse. Thickness of each paw was measured using a pair of digital slide calipers. On day 36, the ankle joints were harvested and examined radiographically and histologically. The bilateral second-to-fourth metatarsophalangeal (MTP) joints were assessed radiographically as follows: 0 = not obvious, 1 = marginal osteoporosis, and 2 = erosion. This system yields a possible score between 0 and 4 per animal. The hind paw of each mouse was dissected and examined histologically after hematoxylin and eosin staining. The severity of arthritis was evaluated according to synovial inflammation, as follows: 0 = no inflammation, 1 = focal inflammatory infiltration, and 2 = severe and diffuse inflammatory infiltration. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

The harvested splenocytes ($1 \times 10^6$ cells) on day 36 were cultured in 48-well plates in Roswell Park Memorial Institute 1640 medium (Sigma-Aldrich) with 10% FBS supplemented with 50 μg/ml denatured (100°C, 10 min) CII. After 72 h, the concentrations of interferon (IFN)-γ and IL-17 in the culture supernatant were measured using ELISA kits (DuoSet; R&D Systems).

Serum samples were obtained on day 36 for measurement of IgG1 anti-CII antibody by ELISA (normal, n = 4; vehicle, n = 12; 4 mg/kg JWH133, n = 12) as described previously [19].

### Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). Immunohistological score was analyzed by student's t-test. Concentration of inflammatory mediators and osteoclastogenesis were analyzed by Kruskal-Wallis test and Dunnett's test. Overtime analysis of arthritis score and paw thickness was performed by 2 way-ANOVA, and point-by-point analysis was followed by student's t-test. Histological and radiographic score, and concentration of IgG1 were analyzed by Kruskal-Wallis test and student's t-test.

## Results

### $CB_2$ expression in the RA synovial tissues and cells

The RA and OA synovial tissues were examined for $CB_2$ expression with immunohistochemical staining. We found positive staining in the lining, and sub-lining layer (Figure 1A), and follicle-like aggregates (Figure 1B). In contrast, minimal staining was observed in the OA synovial tissues (Figure 1C). No signal was observed on specimens stained with an isotype-matched IgG control of irrelevant specificity (Figure 1D). The $CB_2$ staining of 3 RA and 3 OA samples was evaluated with immunohistological score. The scores of $CB_2$ staining of RA samples was significantly higher than that of OA samples (Figure 1E).

In consistence with the fact that macrophages and lymphocytes express $CB_2$ [20,21], immunofluorescence double staining of the RA synovial tissues revealed $CB_2$ expression on the synovial $CD68^+$ macrophages, $CD4^+$ T cells, $CD8^+$ T cells, $CD21^+$ B cells and $vimentin^+$ fibroblast-like appearance cells (Figure 1F).

### Inhibition of inflammatory mediator production from RA FLS with a selective $CB_2$ agonist

FLS produce various inflammatory mediators that play important roles in development of RA. $CB_2$ expression on *in vitro* cultured RA FLS was confirmed with Western blot analysis (Figure 2A), which agreed with previous observation [22]. We evaluated effects of JWH133, a selective $CB_2$ agonist, on the production of IL-6, MMP-3, and CCL2 from FLS stimulated with TNF-α. TNF-α treatment enhanced production of these mediators from RA FLS, which was suppressed by JWH133 dose-dependently (Figure 2B-D). Treatment with JWH133 did not inhibit the proliferation of FLS evaluated with the cell counting kit (Dojindo, Kumamoto, Japan) using WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (data not shown).

### Inhibition of osteoclastogenesis of peripheral blood monocytes with a selective CB2 agonist

In the following experiments, we used human peripheral blood $CD14^+$ monocytes, which have been shown to express $CB_2$ [23], since it is hard to prepare a large number of synovial macrophages. Incubation of peripheral blood $CD14^+$ monocytes with M-CSF and RANKL promoted development of TRAP-positive multinucleated cells. Co-incubation with JWH133 suppressed osteoclast formation dose-dependently (Figure 3A and B). M-CSF and RANKL treatment induced resorption of coated calcium. This was inhibited by the co-incubation with JWH133 dose-dependently (Figure 3C and D). These data showed that JWH133 inhibited the formation and function of human osteoclasts.

### Treatment of murine CIA with JWH133

The inhibitory effects of JWH133 on RA FLS and human osteoclasts prompted us to examine the compound for the inhibitory effects on murine CIA, an animal model of RA. Intraperitoneal administration of JWH133 (1 mg/kg/day or 4 mg/kg/day) or vehicle twice daily was initiated two weeks after the first immunization and continued for 21 days. Treatment with JWH133 (4 mg/kg/day) ameliorated clinical severity of the arthritis (Figure 4A and B). However, the incidence of arthritis was 100% in all groups. On day 36, the ankle joints were harvested and examined histologically and radiographically. Cell infiltration in the synovial tissues and radiographical bone destruction were observed in vehicle-treated CIA mice, and reduced significantly in the JWH133-treated mice (Figure 4C,D,E and F).

Splenocytes from the mice at day 36 were stimulated with CII and the production of IFN-γ and IL-17 was measured. IFN-γ and IL-17 production was upregulated by the CII stimulation. The treatment with JWH133 did not alter the cytokine production (Figure 4G and H). To determine the effect of JWH133 on anti-CII antibody production, we measured serum anti-CII IgG1 antibody titer by ELISA on day 36. While anti-CII IgG1 antibody was not detected in normal mice, it was detected in CIA mice. The treatment with JWH133 significantly lowered anti-CII IgG1 antibody level (Figure 4I). Although IgG2a and IgG2b anti-CII antibodies were also detected in CIA mice, treatment with JWH133 did not significantly alter the levels of antibodies (data not shown).

To examine the therapeutic effects of JWH133 after the onset of the arthritis, we treated the CIA mice with 4 mg/kg JWH133 from day 28 to day 35, and observed inhibition of the arthritis (Figure 5A and B).

## Discussion

The present studies demonstrated that the selective $CB_2$ agonist, JWH133 inhibited production of IL-6, MMP-3 and CCL2 from FLS and M-CSF and RANKL-induced osteoclastogenesis of monocytes/macrophages. Administration of JWH133 ameliorated arthritis severity and bone destruction, and decreased anti-CII IgG1 antibody in a murine model of RA. This effectiveness could

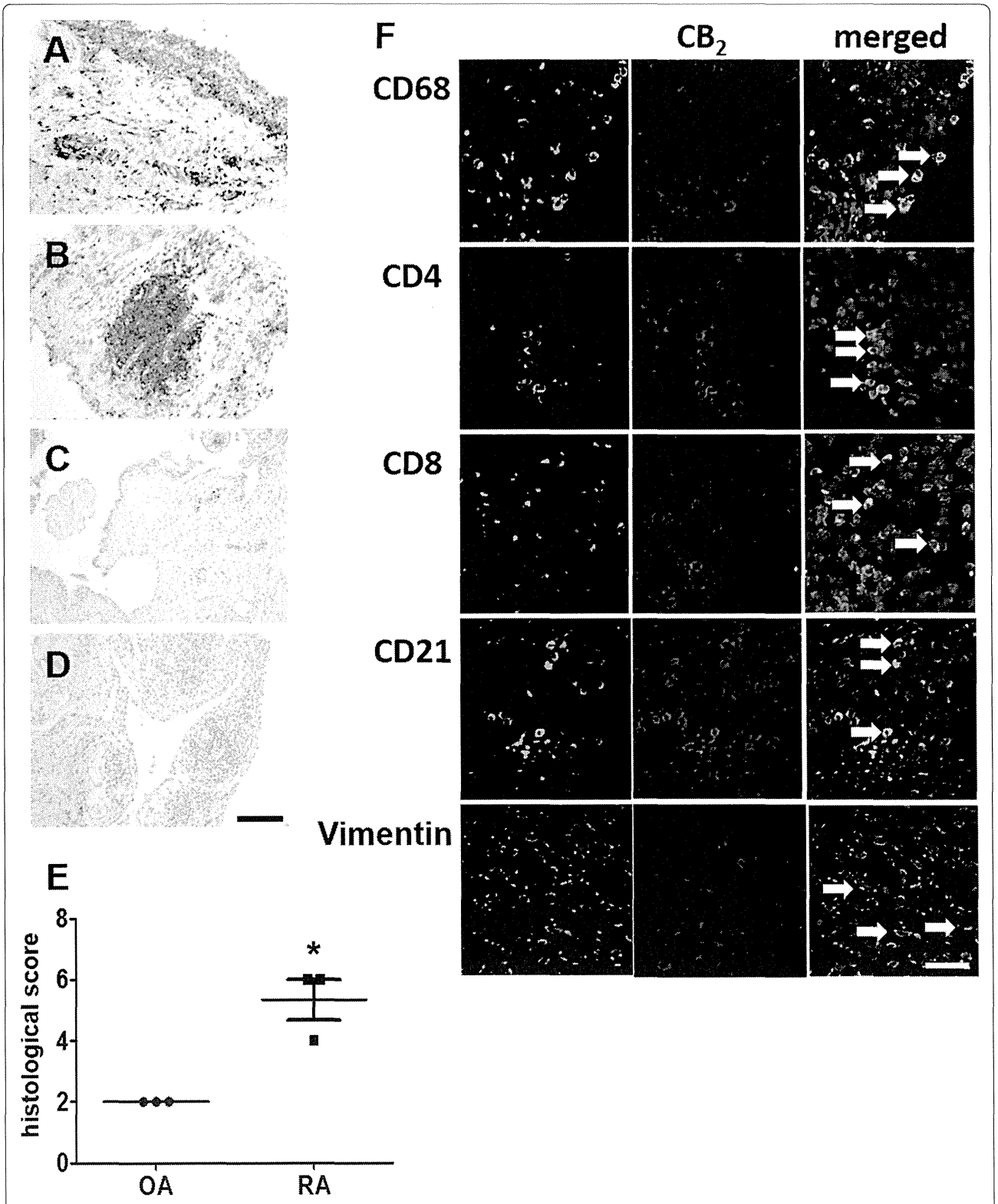


**Figure 1 $CB_2$ expression in the RA synovial tissues.** Synovial tissue samples from patients with RA **(A, B, D)** or with OA **(C)** were stained with anti-$CB_2$ pAb **(A B, and C)**, or normal rabbit IgG **(D)**. All sections were counterstained with hematoxylin. Bar, 400 μm. The representative figures of three RA and three OA synovial tissues are depicted. The severity of the $CB_2$ staining of samples was shown **(E)**. Double immunofluorescence staining of the RA synovial tissues with $CB_2$ and CD68, CD4, CD8, CD21 or vimentin, and DAPI are shown **(F)**. Arrows indicate double-positive cells in the merged image. Bar, 40 μm. *$p < 0.05$.

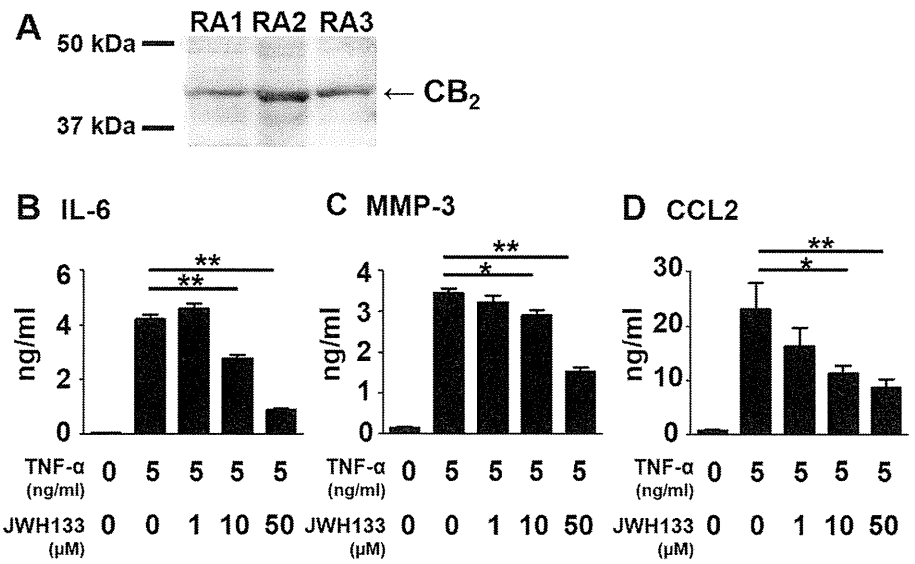


**Figure 2 Inhibition of IL-6, MMP-3, and CCL2 production from FLS by the selective $CB_2$ agonist JWH133.** $CB_2$ expression on *in vitro* cultured FLS from three RA patients was detected with Western blotting analysis **(A)**. Bars indicate 50 and 37 kDa. Predicted molecular weight of $CB_2$ is 45 kDa. RA FLS were treated with various concentrations of JWH133, from 30 minutes before the stimulation with TNF-α for 24 h. Concentrations of IL-6 **(B)**, MMP-3 **(C)**, and CCL2 **(D)** in the cultured supernatants were measured with ELISA. Data is presented as means ± SEM of one of three independent experiments analyzed in quadruplicate. *p < 0.05, **p < 0.01.

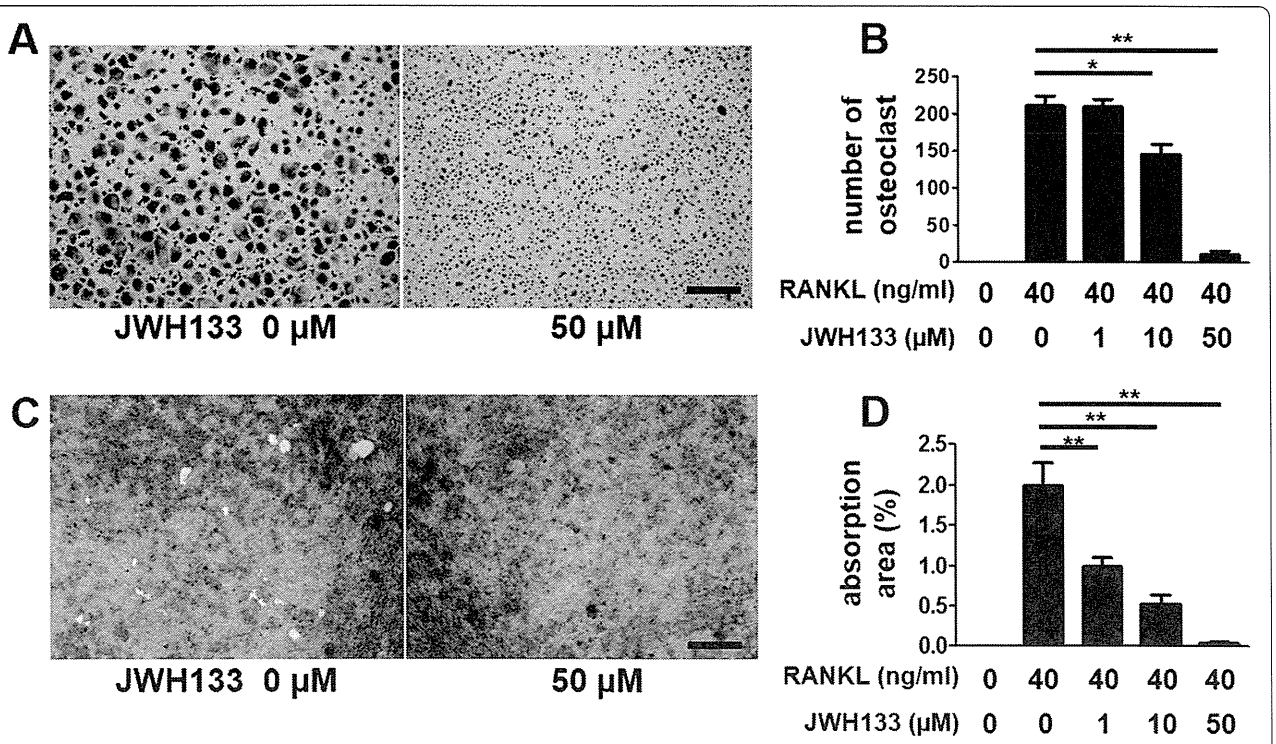


**Figure 3 Effects of the selective $CB_2$ agonist JWH133 on peripheral blood monocytes.** Peripheral blood monocytes were treated with various concentrations of JWH133, from 30 minutes before the stimulation with M-CSF and RANKL. Cells were stained with TRAP **(A)**. TRAP-positive multinucleated cells in a randomly selected field examined at ×40 magnification were counted **(B)**. Representative data (mean ± SEM) from one of three independent experiments analyzed in triplicate are shown. The osteoclasts were incubated on calcium phosphate-coated plates **(C)**. The area of resorption lacunae was examined under light microscopy **(D)**. Representative data (mean ± SEM) from one of three independent experiments analyzed in triplicate are shown. Bar, 400 μm. *p < 0.05, **p < 0.01.

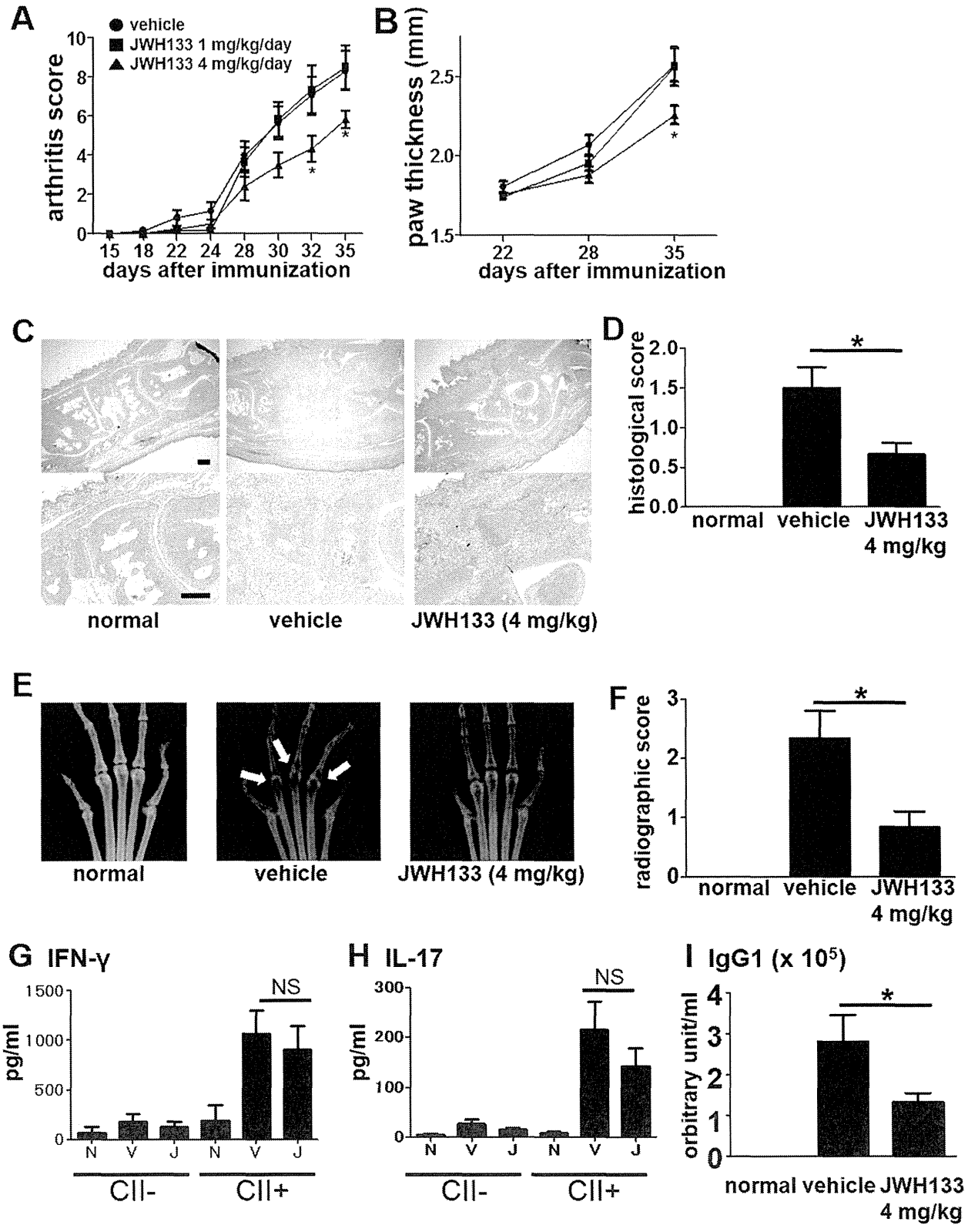


**Figure 4** (See legend on next page.)

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**Figure 4 Effects of the selective $CB_2$ agonist, JWH133 on CIA mice.** JWH133 (1 mg/kg/day or 4 mg/kg/day) or vehicle (n = 12 each) was injected intraperitoneally twice daily from day 15 to day 35. The arthritis severity was recorded as the arthritis score **(A)** and paw thickness **(B)**. Overtime analysis of arthritis score and paw thickness by 2-way ANOVA revealed significant difference between vehicle group and 4 mg/kg/day grope ($p < 0.01$). Representative hematoxylin and eosin staining of ankle joints from normal mice and from mice with CIA after the treatment with vehicle or JWH133 (4 mg/kg/day) are shown **(C)**. Bar, 200 μm. Inflammatory cell infiltration in the ankle joints was evaluated with the histological score **(D)**. Representative radiographs of the ankle joints of normal mice and CIA mice treated with vehicle or JWH133 (4 mg/kg/day) **(E)**. Arrows indicate bone erosion. Bone erosion in the bilateral MTP joints was evaluated with the bone destruction score **(F)**. Splenocytes from normal mice and CIA mice treated with vehicle or JWH133 (4 mg/kg/day) were cultured with CII for 72 h. Concentrations of IFN-γ **(G)** and IL-17 **(H)** in the cultured supernatant were measured by ELISA. N: normal, V: vehicle, J: JWH133, NS: not significant. Serum samples were obtained at day 36 from normal mice and CIA mice treated with vehicle or JWH133 (4 mg/kg/day), and anti-CII IgG1 antibody level was measured by ELISA **(I)**. Values are the mean ± SEM. *$p < 0.05$ versus vehicle.

be attributable to the suppression of inflammatory mediator secretion from FLS and osteoclastogenesis and autoantibody production. Thus, selective $CB_2$ agonists could be a therapeutic agent for RA.

While medical use of cannabinoids such as sedation and analgesia was recorded in 19$^{th}$ century [24], narcotic addiction was always concerned. Recent identification of cannabinoid receptors revealed two distinctive receptors, $CB_1$ and $CB_2$, and putative third receptor, GPR55. Some cannabinoids also act as agonists of TRPV-1, -4 and PPARs. It is expected that selective $CB_2$ agonists act as therapeutic agents that modulate immune functions without any psychoactive effects. JWH133 was applied to our study since it has high selectivity for $CB_2$ against $CB_1$ and has no affinity for GPR55, TRPV-1, -4 or PPARs.

In RA patients, FLS expresses hyperplastic, inflammatory, cartilage- and bone-destructive phenotypes. Cytokines, chemokines and MMPs are secreted by FLS. It was reported that MMP-3 production from FLS stimulated with TNF-α or IL-1β was suppressed by ajulemic acid, a synthetic cannabinoid [22]. However, the receptors of this compound have not been identified. In this study, we showed that the selective $CB_2$ agonist, JWH133 significantly reduced the production of IL-6, MMP-3, and CCL2 from FLS stimulated with TNF-α.

IL-6 has a wide range of functions on immune cells, and plays an important role in RA [25]. Blockade of IL-6 signaling by anti-IL-6 or -IL-6 receptor mAbs is effective treatment [26,27]. Among various chemokines, only CCL2 gene expression is reportedly higher in RA FLS than in OA FLS [28]. Monocyte migration induced by RA stromal cell line supernatants was blocked with anti-CCL2 mAbs [29]. MMP-3 was highly expressed in the RA pannus tissues [30]. In the cytoplasm, $CB_2$ stimulation leads to inhibition of adenylyl cyclase and subsequent decrease of the intracellular cAMP level. It results in decreased activity of protein kinase A and transcription factors such as NF-κB and NFAT [31,32]. Thus, the treatment of JWH133 could suppress other inflammatory mediators than IL-6, CCL2 and MMP-3, which are involved in pathology of the arthritis.

JWH133 may affect other types of cells involved in the arthritis. RANTES/CCL5-induced chemotaxis of macrophages was inhibited with delta-9-tetrahydrocannabinol, the major component in *marijuana*, acting through $CB_2$ [33]. Anandamide, one of endocannabinoids, suppressed IL-17 production from Th17 cells primarily via $CB_2$ [34]. JWH015, another selective $CB_2$ agonist, inhibited CXCL12-induced chemotaxis of T cells [35]. We found that $CB_2$ was expressed broadly in the RA synovial cells including macrophages, T cells, and B cells.

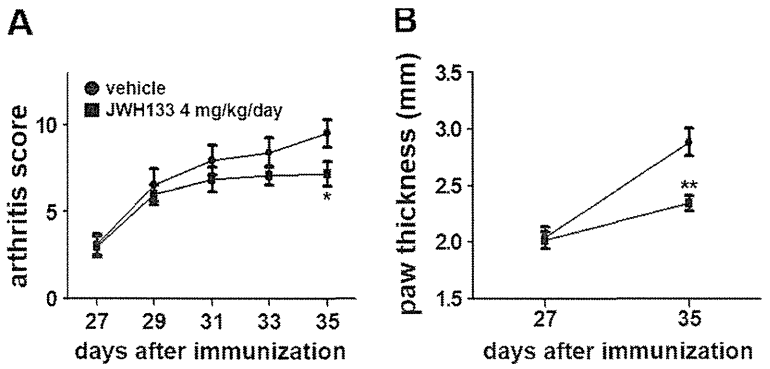


**Figure 5 Therapeutic effect of JWH133 on CIA.** CIA mice were treated with intraperitoneal injection of JWH133 (4 mg/kg/day) or vehicle (n=12 each) twice daily from day 28 to day 35, after the onset of the disease. The arthritis severity was recorded as the arthritis score **(A)** and paw thickness **(B)**. Overtime analysis of arthritis score and paw thickness by 2-way ANOVA revealed significant difference between vehicle group and 4 mg/kg/day grope ($p < 0.01$). Values are the mean ± SEM. *$p < 0.05$, **$P < 0.01$ versus vehicle.

Suppression of these cells could contribute to suppression of the arthritis.

It was reported that $CB_2$-deficient mice develop osteoporosis with age [36]. HU308, another selective $CB_2$ agonist, inhibited osteoclast formation of RANKL-stimulated RAW264.7 cells as well as bone marrow cells from normal mice but not from $CB_2$-deficient mice [36]. In agreement with this observation, we demonstrated that JWH133 inhibited osteoclastogenesis of human peripheral blood monocytes and bone destruction of CIA mice. Since bone destruction is often a serious issue in RA patients as well as chronic pain and can result in functional decline, inhibitory effect of selective $CB_2$ agonists for osteoclastogenesis could be another feature in RA treatment.

In this study, we observed amelioration of CIA with JWH133. Since T helper cell differentiation influences the development of CIA [37,38], we measured the production of IFN-γ and IL-17 by CII-stimulated splenocytes from the CIA mice. No significant difference among the groups was revealed in our study. It is suggested that the treatment with JWH133 did not affect Th1 and Th17 differentiation, which may not be attributable to the amelioration of CIA. On the other hand, the treatment with JWH133 decreased the level of serum anti-CII IgG1 antibody. In the previous study, $CB_2$ mRNA was detected in peripheral B cells [20]. We determined $CB_2$ expression on B cells in RA synovial tissue. Although the effect of $CB_2$ for immunoglobulin production has not been reported, the administration of JWH133 directly or indirectly may affect B cells to suppress anti-CII IgG1 antibody production of CIA mice and contribute to the amelioration of the arthritis.

This is the first report of therapeutic effect of a selective $CB_2$ agonist on CIA. Although the effect was mild, optimization of dosage and/or treatment protocol might enhance the effect. Perhaps, more potent selective $CB_2$ agonists might solve this problem.

## Conclusions

We demonstrated that JWH133, the selective $CB_2$ agonist, provides clinical effectiveness against CIA mice probably through the immunosuppressive effects for FLS and monocytes and inhibition of anti-CII Ab production. Addition to the analgesic effect as previously reported, selective $CB_2$ agonists could be a new therapy for RA.

### Abbreviations

CB: Cannabinoid; $CB_1$: Cannabinoid receptor 1; $CB_2$: Cannabinoid receptor 2; CCL2: CC chemokine ligand 2; CFA: Complete Freund's adjuvant; CIA: Collagen-induced arthritis; CII: Collagen type II; FLS: Fibroblast-like synoviocytes; M-CSF: Macrophage colony-stimulated factor; MMP: Matrix metalloproteinase; MNC: Multinucleated cells; OA: Osteoarthritis; RA: Rheumatoid arthritis; RANKL: Receptor activator of nuclear factor kappa-B ligand; TRAP: Tartrate-resistant acid phosphatase.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

SF participated in the design of the study, carried out the experiments and statistical analysis, and drafted the manuscript. AT, WY, CM and YM assisted carrying out the experiments and manuscript preparation. HK, MH, NM and TN conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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CASE REPORT

# Successful treatment of eosinophilic granulomatosis with polyangiitis (EGPA; formerly Churg–Strauss syndrome) with rituximab in a case refractory to glucocorticoids, cyclophosphamide, and IVIG

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**Abstract**

A 44-year old woman with eosinophilic granulomatosis with polyangiitis (EGPA) developed sequential paralysis of different cranial nerves despite treatments including methylpredonisolone pulse therapy, intravenous immunoglobulins (IVIG), and cyclophosphamide. Infusions of rituximab ameliorated her neurological symptoms and serological inflammatory findings. Rituximab, a specific B cell-targeting therapy, might offer an alternative for refractory EGPA with possible advantages of cost and ease of use compared to IVIG, which also targets (at least in part) B lymphocytes and immunoglobulin production.

**Keywords**

Eosinophilic granulomatosis with polyangiitis, IVIG, Rituximab

**History**

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## Introduction

Eosinophilic granulomatosis with polyangiitis (EGPA) (formerly Churg–Strauss syndrome) is a systemic granulomatous vasculitis with eosinophilia in a patient with a history of allergic disease. The involvement of small to medium vessels is characteristic of EGPA, as is the presence of antineutrophil cytoplasmic antibody (ANCA) in the serum. Both of these features qualify EGPA for inclusion as an ANCA-associated vasculitis (AAV).

Although high-dose glucocorticoids (GC) with cyclophosphamide (CPA) has been the main treatment applied in severe cases of EGPA, about 10 % of these cases have been found to be treatment resistant [1]. Further therapeutic options have been sought for such cases, and successful induction of remission has been reported with the use of both intravenous immunoglobulins (IVIG) and rituximab (RTX) [2]. However, repeated administration and/or combination therapies are sometimes required to achieve maximum benefit [3].

Rituximab (RTX) is a chimeric anti-CD20 monoclonal antibody that has been approved for use in cases of lymphoid malignancy, rheumatoid arthritis, and, more recently, microscopic polyangiitis (MPA) and granulomatosis with polyangiitis (GPA). Although it has proven to be efficacious for AAV in two randomized controlled studies [4, 5], its efficacy in EGPA cases has remained unclear.

We hereby present a case of EGPA resistant to both CPA and IVIG, which was successfully treated with RTX.

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## Case report

A 44-year-old woman with a history of bronchial asthma was admitted to a local hospital in June 2011 for fever with numbness and weakness of her extremities. Laboratory data showed peripheral eosinophilia (6579/μl) and elevated CRP (34.5 mg/l), as well as the presence of MPO–ANCA (180 EU/l). Nerve conduction test revealed low amplitude in the right median, the left ulnar, and the right sural nerves, suggestive of mononeuritis multiplex. She was diagnosed with EGPA, and treatment was initiated with intravenous pulses of methylprednisolone.

Peripheral blood eosinophil count and MPO–ANCA normalized within six weeks during the oral prednisolone (PSL) taper. However, she remained febrile, her neurological symptoms persisted, and her CRP remained elevated (Fig. 1). A trial of IVIG (400 mg/kg/day over five days) was ineffective, with a new left facial nerve paralysis developing during the second course. One intravenous dose of CPA (500 mg) was administered along with the initiation of trimethoprim-sulfamethoxazole for preventing pneumocystis pneumonia, during which time the facial nerve paralysis worsened. There was no evidence of meningitis or hypertrophic pachymeningitis by cerebrospinal fluid analysis and brain MRI.

At this point (in August), the patient was transferred to our hospital, and she still presented febrile. The physical examination revealed that the cranial nerves other than the left facial nerve were intact and that paresthesia and weakness of her extremities had persisted. Peripheral blood count showed an absence of eosinophils, with total white blood cells 17,200/μl, hemoglobin level 8.2 g/dl, and platelets 469,000/μl. Serum CRP (159 mg/l) and eosinophil cationic protein (ECP; 36 μg/dl) were elevated, while MPO–ANCA remained negative. Serum IgG concentration was 1517 mg/ml (normal range: 868–1780 mg/ml). Methylprednisolone pulses improved the facial nerve palsy and the patient became

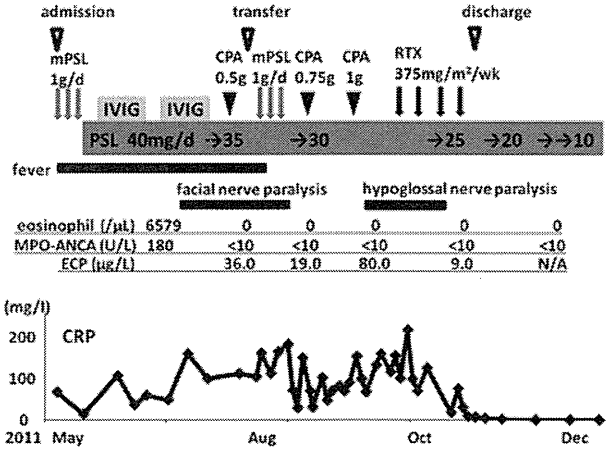


Fig. 1 Clinical course. This EGPA patient was resistant to corticosteroid, IVIG, and CPA. Treatment with RTX ameliorated cranial nerve involvement and reduced serum ECP and CRP levels. She remains in remission with B-cell depletion six months after RTX therapy. *mPSL* methylprednisolone, *PSL* prednisolone, *IVIG* intravenous immunoglobulin, *CPA* cyclophosphamide, *RTX* rituximab, *ECP* eosinophil cationic protein

afebrile, although her CRP remained high. Two additional intravenous doses of CPA (750 mg, followed by 1000 mg) also failed to decrease the CRP. Moreover, the patient started to complain of difficulty swallowing, and paralysis of the right hypoglossal nerve was demonstrated clinically. Serum ECP rose to 80 μg/dl while serum IgG decreased to 801 mg/ml.

Subsequently, RTX was administered at a dose of 480 mg (375 mg/m$^2$) once a week for four weeks under written informed consent. Three weeks after the first dose, the hypoglossal nerve paralysis had disappeared completely, with decreased peripheral B-cell counts of 2 cells/μl as compared to 164 cells/μl before the RTX administration. Serum CRP and ECP diminished to within normal ranges by the time of the fourth dose, although the peripheral neuropathy at the extremities did not improve. There was no occurrence of adverse events despite the depletion of peripheral B cells and the low serum IgG concentration (760 mg/ml). At six months after completing treatment with RTX, the patient remained in remission on PSL 10 mg/day without peripheral B-cell recovery.

## Discussion

Rituximab showed dramatic efficacy in our patient who had EGPA that was resistant to both conventional treatment and IVIG. The success obtained with RTX in the present case is consistent not only with the demonstrated efficacy of RTX in cases of MPA and GPA [4, 5], but also with the results achieved when it was used in eleven previously reported cases (Table 1) [6–11]. Among these 12 cases (including the case herein), three were resistant to previous IVIG, and at least five achieved remission without concomitant immunosuppressants or high-dose corticosteroids, suggesting a possible advantage of RTX in remission induction. As IVIG is presumed to exert its efficacy partly by providing a negative feedback signal on B cells mediated through FcγRIIB [12], the depletion of B cells using RTX may be a more potent and direct mechanism.

The efficacy of IVIG in EGPA has been previously demonstrated in two nonrandomized interventional studies. Tsurikisawa et al. [13] reported that IVIG showed significantly greater improvement of muscle weakness, dysesthesia, and cardiac output in 22 EGPA patients refractory to conventional treatment compared to 24 patients who did not receive IVIG. In a separate report, Danieli et al. [14] found that repeated IVIG combined with plasmapheresis in addition to conventional treatment achieved a significantly higher remission rate (100 %) than conventional treatment alone (44 %) in newly diagnosed EGPA. Based on these findings, IVIG can be recommended in particular for cases with persistent neurological deficits and/or cardiac dysfunction, as well as for difficult-to-treat cases. The efficacy of IVIG monotherapy for inducing remission remains unproven.

Although the potential clinical superiority of RTX needs to be demonstrated in larger studies, its economic and logistical advantages are clear. The administration of IVIG requires hospitalization, which sometimes needs to be repeated. Furthermore, the clinical status of the patient may dictate the need for concomitant plasma exchange, significantly increasing the cost of treatment. Logistically, the availability of IVIG has been problematic globally, and the use of alternative treatments, when possible, has

Table 1. Cases of EGPA successfully treated with RTX

| Patient no. [reference] | Age, sex | Involved organs | ANCA | Previous treatments (other than GC) | Concomitant treatments with RTX | Observation period after RTX administration/relapse | Additional RTX use/its indication |
|---|---|---|---|---|---|---|---|
| 1 [6] | 49, M | Kidney, skin | PR3 | IVCY, AZA | PSL 30 mg/day | 3 months/not relapsed | None |
| 2 [7] | 37, F | Myocarditis | (–) | IVCY, IVIG, MMF, alemtuzumab | PSL 15 mg/day | 9 months/not relapsed | At 6 months/ prophylaxis |
| 3 [7] | 37, F | PNS, skin | (–) | CPA, AZA, MMF, alemtuzumab | PSL 10 mg/day | 12 months/relapsed at 6 months | At 6 months/ relapse |
| 4 [8] | 40, M | Lung | PR3 | IVCY, PE | PSL[a] + CPA | 9 months/not relapsed | None |
| 5 [8] | 66, M | PNS | MPO | IVIG, IVCY, PE | Low-dose PSL[a] | 3 months/not relapsed | None |
| 6 [9] | 46, F | CNS | MPO | IVCY, MMF | PSL 5 mg/day + MMF | 4 months/not relapsed | None |
| 7 [10] | 50, M | PNS, skin | (–) | MTX, CyA, AZA IFX, anakinra | PSL[a] | 12 months/not relapsed | At 6 and 12 months/ prophylaxis |
| 8 [10] | 35, F | Lung | (–) | AZA | PSL[a] | 6 months/not relapsed | At 6 months/ prophylaxis |
| 9 [11] | 54, F | Kidney | MPO | IVCY, MTX | PSL 1 mg/kg/day | 12 months/relapsed at 6 months | At 6 months/ relapse |
| 10 [11] | 54, F | Kidney, PNS | MPO | (–) | PSL 1 mg/kg/day | 12 months/not relapsed | None |
| 11 [11] | 65, M | Kidney, PNS | MPO | (–) | PSL 1 mg/kg/day | 12 months/not relapsed | None |
| 12 (present case) | 44, F | CNS, PNS | MPO | IVIG, IVCY | PSL 25 mg/day | 6 months/not relapsed | None |

*PNS* peripheral nervous system, *CNS* central nervous system, *IVCY* intravenous CPA pulse therapy, *IVIG* intravenous immunoglobulin, *AZA* azathioprine, *MMF* mycophenolate mofetil, *CPA* oral cyclophosphamide, *PE* plasma exchange, *MTX* methotrexate, *CyA* cyclosporine A, *IFX* infliximab

[a]The dosage of PSL was not available

been recommended. Compared with IVIG, one course of RTX may provide sustained efficacy for approximately six months or longer, with at least some of the infusions possible in an outpatient setting.

As pharmacotherapeutic decisions need to be made by weighing up the balance of efficacy and safety, the risk of progressive multifocal leukoencephalopathy (PML) needs to be considered specifically for RTX. Fortunately, these cases are rare; most of them are associated with either previous or concomitant exposure to other immunosuppressive agents. Still, when compared to the safety of IVIG, the risk of developing serious infections should be an important consideration with RTX.

RTX could be an alternative for remission induction in EGPA cases. However, it remains unclear whether the scheduled RTX treatment or treatments with other oral immunosuppressants should be followed as maintenance therapy. In the present case, azathioprine was used successfully, in accordance with the EULAR recommendation for maintenance therapy after the conventional remission induction treatments [2]. An ongoing randomized controlled trial of RTX versus azathioprine as maintenance therapy (MAINRITSAN) will provide us with important information.

In general, peripheral eosinophil counts as well as serum ECP levels serve as biomarkers of the disease activity of EGPA [15, 16]. In the present case, the ECP level reflected the disease activity better than the eosinophil count. Together with another report that described a discrepancy between the levels of these two biomarkers [17], our observation suggests that serum ECP might be the more sensitive biomarker.

In conclusion, RTX is a potent alternative therapy for refractory EGPA. Further clinical investigations, especially with larger numbers of patients, are needed to confirm its efficacy and safety, as well as its most appropriate position in the therapeutic armamentarium.

## Conflict of interest

PYS is currently employed by UCB Japan, Co., Ltd. All other authors have declared no conflict of interest.

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ORIGINAL ARTICLE

# A comparison of incidence and risk factors for serious adverse events in rheumatoid arthritis patients with etanercept or adalimumab in Korea and Japan

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**Abstract**

*Objective.* To compare the incidence and risk factors of serious adverse events (SAEs) in rheumatoid arthritis (RA) patients treated with etanercept (ETN) or adalimumab (ADA) between Korean and Japanese registries.

*Methods.* We recruited 416 RA patients [505.2 patient-years (PYs)] who started ETN or ADA from Korean registry and 537 RA patients (762.0 PY) from Japanese registry. The patient background, incidence rate (IR) of SAE in 2 years, and risk factors for SAEs were compared.

*Results.* Korean patients were younger and used more nonbiologic DMARDs, higher doses of methotrexate, and lower doses of prednisolone (PSL). The IR of SAEs (/100 PY) was higher in the Japanese registry compared to the Korean [13.65 vs. 6.73]. In both registries, infection was the most frequently reported SAE. The only significant risk factor for SAEs in Korean registry was age by decade [1.45]. In Japanese registry, age by decade [1.54], previous use of nonbiologic DMARDs $\geq 4$ [1.93], and concomitant use of oral PSL $\geq 5$ mg/day [2.20] were identified as risk factors for SAEs.

*Conclusions.* The IR of SAE in Japan, especially infection, was higher than that of Korea, which was attributed to the difference of demographic and clinical characteristics of RA patients and treatment profiles.

**Keywords**

Epidemiology, Registry, Rheumatoid arthritis, Safety

**History**

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## Introduction

The introduction of biologic disease-modifying antirheumatic drugs (biologic DMARDs) in the past decade has revolutionized treatment of rheumatoid arthritis (RA). Efficacy and safety of treatment with biologic DMARDs have been demonstrated in a number of clinical trials, but cost and long-term effectiveness of treatment with biologic DMARDs and safety in older patients or those with comorbidities, who are generally excluded from clinical trials, have been of concern [1]. To complement the evidence obtained from clinical trials, observational cohorts for RA patients treated with biologic DMARDs have been established in many countries, and have provided indispensable evidence for the safety and effectiveness of biologic DMARDs in clinical practice. However, some cohorts have reported results with differing magnitudes or even discordance of risk for the same adverse events [2]. For example, the incidence of serious infections in European RA registries was comparable [3,4], whereas in the US, lower rates have been reported in some studies [5]. These discrepant results arise from methodological differences, such as case definition for adverse events, length of follow-up, or selection and structure of a comparator group. Difference in treatment profile and ethnics may also account for the discrepancy. Therefore, a careful comparison of registries from various point of views including methodology is

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imperative to understand similarities and differences in the results obtained from each registry [6].

Through international collaborations among countries, the comparison of data from RA patients treated with biologic DMARDs will allow us to investigate the impact of differences in patients' characteristics and health care systems on efficacy and safety of the treatment. Curtis et al. [2] have conducted the qualitative comparison of RA biologics registries in US and Europe and reported that different patients' demographics, patterns of comorbidities, and sociodemographic characteristics provide valuable information to address the comparative safety of treatments for RA. However, no international collaborative studies have yet been reported to investigate the same outcomes using harmonized methodologies. In Korea, the effectiveness and safety of biologic DMARDs in clinical practice have been reported using a retrospective biologic DMARDs registry (REtrospective study for Safety and Effectiveness of Anti-RA treatment with biologiCs, RESEARCh) [7]. In Japan, the REgistry of Japanese rheumatoid Arthritis patients on Biologics for Long-term safety (REAL) has provided evidence about safety of biologic DMARDs in Japanese RA patients [8,9]. Taking advantage of these established cohorts for patients with RA, we conducted the first epidemiological study to compare data from two countries where biologic DMARDs are widely used for treatment of RA.

For this study, we carefully scrutinized features of Korean and Japanese registries and considered standardization of methodological approaches. We conducted this study to reveal the factors influencing safety of adalimumab (ADA) or etanercept (ETN) by comparing RA patients treated with these drugs from Korean and Japanese registries in terms of retention rates and reasons for discontinuation of biological DMARDs, incidence rates (IR) of serious adverse events (SAEs), and factors influencing their development.

## Patients and methods

### Database and patients

*RESEARCh*

The retrospective registry of Korean patients with RA, the RESEARCh, was established to evaluate the safety and effectiveness of biologic DMARDs by Clinical Research Center of Rheumatoid Arthritis (CRCRA) funded by Ministry of Health and Welfare, Republic of Korea [7]. All patients meeting the 1987 American College of Rheumatology criteria for RA who had ever been treated with biologic DMARDs from December 2000 to June 2011were identified from the medical records of Hanyang University Hospital for Rheumatic Diseases. The RESEARCh study was approved by the ethics committees of the Hanyang University Hospital, and informed consent was not required because the data was deidentified and collected retrospectively.

Comprehensive chart reviews for all patients were undertaken by well-trained health professionals; and demographics, disease activity, comorbidities, medications, and laboratory data during the use of biologic DMARDs and their SAEs were collected. For the patients who were in use of biologic DMARDs at the time of data collection, the observational period was defined from starting point of current agent to assessment date. For the other patients who had stopped biologic DMARDs before data collection, the agent with longest use for each patient was included in this database. Demographic features of RA patients and the persistence of TNF inhibitors in the RESEARCh database were quite similar to those of a previously reported study using nation-wide claims database of Korea; mean age ($50.5 \pm 13.2$ in the RESEARCh vs. $50.6 \pm 14.9$ in the nation-wide database), proportion of female (86.1% vs. 84.9%), and persistence of TNF inhibitors during one year (74% vs. 73%) [7,10].

*REAL*

REAL is a prospective cohort established to investigate the long-term safety of biologic DMARDs in RA patients. Twenty-seven institutions participate, including 16 university hospitals and 11 referring hospitals. Details of the REAL have been previously described [9,11]. Briefly, the criteria for patient enrollment in the REAL include meeting the 1987 American College of Rheumatology criteria for RA, written informed consent, and starting or switching treatment with biologic DMARDs or starting, adding, or switching nonbiologic DMARDs at the time of enrollment in the REAL. Demography, disease activity, comorbidities, treatments, and laboratory data at the time of enrollment in the REAL were recorded. A follow-up form was submitted every 6 months by participating physicians to the REAL Data Center at the Department of Pharmacovigilance of Tokyo Medical and Dental University to report the occurrence of SAEs, current RA disease activity, treatments, and clinical laboratory data. Each patient is followed for 5 years. Enrollment in the REAL database was started in June 2005 and closed in January 2012. Data were retrieved from the REAL database on August 24, 2011 for this study. The REAL study was approved by the ethics committees of the Tokyo Medical and Dental University Hospital and other participating institutions. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983.

### Patients and follow-up

We first identified 416 Korean RA patients whose registered biologic DMARDs in the RESEARCh database were ADA or ETN and 537 Japanese patients with RA who used ADA or ETN as the first biologic DMARDs in the REAL database, and enrolled themselves in this study. The reason for selecting ADA and ETN for this study is that these two biologics were approved within two calendar years in both countries. The observation period for this study started at the first dose of one of these biologic DMARDs. Observation of each patient was stopped either 2 years after the start of the observation period, or on the date of discontinuation of these biologic DMARDs, switching to other biologic DMARD, death, loss-to-follow up, or enrollment in clinical trials, whichever came first. We defined discontinuation of treatment with ADA or ETN as stopping administration of these agents for more than 90 days. Reasons for discontinuation of these biologic DMARDs were retrieved from medical records and classified into adverse events (AEs), Lack of efficacy (LOE), or miscellaneous. When a patient had two or more reasons for drug discontinuation, site investigators assigned precedence and the primary reason contributing to drug discontinuation for the patient was used.

### Definition for comorbidity

For qualitative comparison, comorbidity was defined as cardiovascular and cerebrovascular diseases, including angina, myocardial infarction, heart failure, and strokes; pulmonary diseases, including interstitial lung diseases, chronic obstructive pulmonary diseases, and asthma; or liver diseases, including abnormalities in liver function tests, liver cirrhosis, hepatitis B, and hepatitis C. Renal dysfunction was defined using the estimated glomerular filtration rate (eGFR). We used a modification of diet in renal disease (MDRD) formula to calculate eGFR and categorized according to the stage of chronic kidney disease (CKD) [12]. Anemia was defined using the WHO criteria (hemoglobin level $< 13$ g/dl for men and $< 12$ g/dl for women) [13].

### Definition of SAEs

Our definition of a SAE, including serious infection (SI), was based on the report by the International Conference on Harmonization. In addition, bacterial infections that required intravenous administration of antibiotics, as well as opportunistic infections, were also regarded as SAEs. SAEs were classified using the System Organ Class (SOC) of the medical dictionary for regulatory activities (MedDRA version 11.1). SAEs were attributed to ETN or ADA when they developed during treatment with these biologics and no risk window was applied.

### Statistical analysis

The chi-square test was used for comparison of categorical variables and the Mann–Whitney test for continuous variables. Drug retention rates were compared using the Kaplan–Meier method and the log-rank test. Crude IRs per 100 PY and crude incidence rate ratios (IRRs) with their 95% confidence intervals (CI) comparing Japan to Korea were calculated for all SAEs occurring from the first dose of ADA or ETN to the end of the observation period. For multivariate analysis, the Cox regression model with the forced entry method was employed. These statistical analyses were performed using SPSS (version 20.0, SPSS Inc., Chicago, IL USA). All $p$ values were 2-tailed and $p < 0.05$ was considered statistically significant.

## Results

### Demographic and clinical baseline characteristics of patients from the two registries

We first compared baseline demographic and clinical characteristics of RA patients who used ADA or ETN in each registry (Table 1 and Figure 1). Patients in the RESEARCh were younger ($47.5 \pm 15.8$ vs. $58.9 \pm 13.3$ years-old, $p < 0.001$) and had shorter disease duration ($8.5 \pm 6.7$ vs. $9.9 \pm 9.0$, years $p = 0.009$) than those in the REAL. The proportions of patients without previous exposure to biologic DMARDs (i.e., biologic DMARD-naïve patients) did not differ between the two registries, while 60.0% of the patients in the RESEARCh, but only 29.4% in the REAL, experienced four or more nonbiologic DMARDs ($p < 0.001$). The mean numbers of previous nonbiologic DMARDs were 4.1 in the RESEARCh and 2.6 in the REAL; the distribution is shown in Figure 1A. The mean Disease Activity Score calculated based on three variables including 28-swollen and tender joints count and C-reactive protein at starting biologic DMARDs did not differ between the registries. Patients in the RESEARCh used concomitant methotrexate (MTX) more frequently and at higher dosage than those in the REAL (75.9% and $13.3 \pm 3.2$ mg/week vs. 54.2% and $7.7 \pm 2.4$ mg/week, $p < 0.001$ for both) (Table 1 and Figure 1B). On the other hand, patients in the REAL used concomitant corticosteroids (CSs) more frequently and at higher dosage than those in the RESEARCh (PSL-equivalent dose, $6.0 \pm 3.5$ mg/day vs. $4.5 \pm 3.1$ mg/day, $p < 0.001$) (Table 1 and Figure 1C).

The rates for comorbidities differ significantly between the two registries. The rates for patients with peptic ulcer (6.0% for the RESEARCh vs. 0.7% for the REAL), liver disease (10.8% vs. 6.7%), hypertension (21.9% vs. 15.8%), and anemia (73.8% vs. 60.5%) were significantly higher in the RESEARCh compared to the REAL. However, the rates for pulmonary disease (5.3% vs. 20.3%) and diabetes mellitus (9.4% vs.13.6%) were significantly higher in the REAL than in the RESEARCh (Table 1).

Table 1. Demographic and clinical characteristics of patients with RA treated with ETN or ADA from Korean (RESEARCh) and Japanese (REAL) registries.

| | RESEARCh ($n = 416$) | REAL ($n = 537$) | $p$ value |
|---|---|---|---|
| Age (years-old) mean ± SD | 47.5 ± 15.8 | 58.9 ± 13.3 | <0.001 |
| >65, n (%) | 57 (13.7) | 201 (37.4) | <0.001 |
| Gender (female), % | 84.3 | 79.0 | 0.067 |
| Disease duration (years), mean ± SD | 8.5 ± 6.7 | 9.9 ± 9.0 | 0.009 |
| DAS28(3)/CRP*, mean ± SD | 4.4 ± 0.9 | 4.5 ± 1.3 | 0.973 |
| Unexposed to biological DMARDs, (%) | 333 (80.0) | 440 (81.9) | 0.505 |
| Number of previous nonbiologic DMARDs† ≥4, (%) | 249 (60.0) | 158 (29.4) | <0.001 |
| MTX, mg/week (%) | 13.3 ± 3.2 (75.9) | 7.7 ± 2.4 (54.2) | <0.001 |
| Corticosteroid**, mg/week (%) | 4.5 ± 3.1 (72.1) | 6.0 ± 3.5 (67.6) | <0.001 |
| Cardiovascular disease, n (%) | 11 (2.6) | 30 (5.6) | 0.026 |
| Pulmonary disease‡, n (%) | 22 (5.3) | 109 (20.3) | <0.001 |
| Liver disease, n (%) | 45 (10.8) | 36 (6.7) | 0.024 |
| Peptic ulcer, n (%) | 25 (6.0) | 4 (0.7) | <0.001 |
| Diabetes mellitus, n (%) | 39 (9.4) | 73 (13.6) | 0.045 |
| Hypertension, n (%) | 91 (21.9) | 85 (15.8) | 0.017 |
| Anemia¶, n (%) | 307 (73.8) | 325 (60.5) | <0.001 |
| Renal dysfunction§, n (%) | | | |
| Advanced staged CKDs (CKD3, 4 or 5), n (%)§ | 23 (5.5%) | 14 (2.6%) | 0.021 |

SD, standard deviation; DAS28, disease activity score including 28-joint count; CRP, C-reactive protein; DMARDs, disease-modifying antirheumatic drugs; MTX, methotrexate; PSL, prednisolone; CKD, chronic kidney disease; GFR, glomerular filtration rate

*DAS 28(3)/CRP was calculated based on three variables; swollen and tender joint counts and CRP.

**The oral corticosteroid dose was converted to the equivalent PSL dosage.

†Nonbiologic DMARDs included MTX, hydroxycholoroquine, sulfasalazine, leflunomide, bucillamine, mizoribine, tacrolimus, azathioprine, cyclosporin.

‡Pulmonary disease included interstitial lung disease, chronic obstructive pulmonary disease, and asthma.

¶Anemia was defined using WHO criteria.

§Renal dysfunction was defined using GFR calculated by modification of diet in renal disease. GFR was categorized according to the staging of CKD.

MTX and corticosteroid doses are shown as the mean ± SD among users of these drugs.

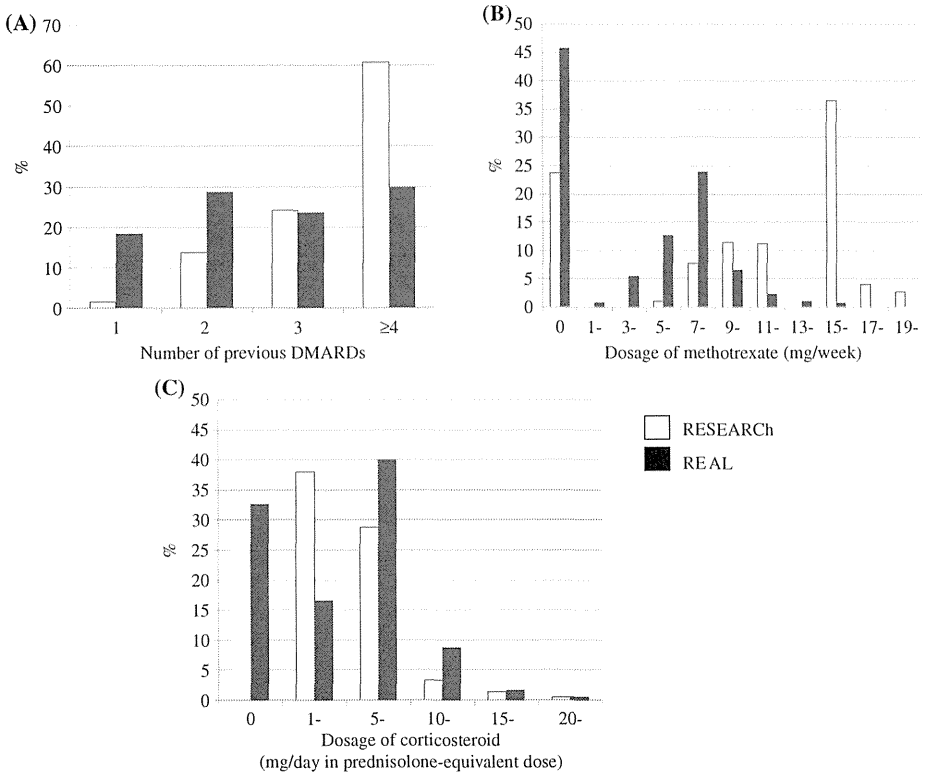


Figure 1. Comparison of RA-related medication usage between two registries, RESEARCh (Korea) and REAL (Japan). For all graphs, the white columns indicate the rates in RESEARCh and the black columns indicate the rates in REAL.
(A) Comparison of the numbers of previous nonbiologic DMARDs between the two registries.
(B) Comparison of baseline MTX dosage between the two registries.
(C) Comparison of baseline corticosteroid dosage between the two registries.



### Reasons for drug discontinuation and retention rates for ADA and ETN

The median interquartile range treatment period for each registry was 1.3 (0.5–2.0) years for the RESEARCh and 2.0 (0.8–2.0) years for the REAL. The numbers of patients who discontinued ADA or ETN for any reasons during the observation period were 124 (29.8%) for the RESEARCh and 144 (26.8%) for the REAL ($p = 0.308$ by chi-square). The reasons for discontinuation of ETN or ADA in each registry are shown in Table 2. The development of AEs was the most frequent reason for the discontinuation in both the RESEARCh ($n = 41$, 33.1%) and the REAL ($n = 56$, 38.9%). The two major AEs leading to discontinuation of the biologic DMARDs were infection and allergic reaction for both registries. There was no significant difference in the retention rates of ETN and ADA for 2 years between the registries (64.6% in the RESEARCh, 70.1% in the REAL, $p = 0.060$ by Kaplan–Meier analysis and log-rank test [supplementary Figure 2A available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2013.860695]), and no significant differences for treatment discontinuation due to AEs ($p = 0.848$ by log-rank test [supplementary Figure 2B available online at http://informahealthcare.com/doi/abs/10.3109/14397595.2013.860695]).

### Types and occurrence of SAEs

Among 416 patients with 505.2 PY in the RESEARCh, 34 SAEs were reported during the observation period, while 104 SAEs in 537 patients with 762.0 PY were found in the REAL. Based on the SAE categories classified using the SOC, 'infection and infestations' was the most common category in both registries (15 cases for the RESEARCh and 38 for the REAL) and 'respiratory, thoracic and mediastinal disorders' was the second most common category (5 cases for the RESEARCh and 13 for the REAL).

Pulmonary infection was the most frequent site-specific infection in both registries (9 cases for the RESEARCh and 22 cases for the REAL), followed by skin infection, including herpes zoster and cellulitis (4 cases for the RESEARCh and 8 cases for the REAL). Other infections included one bone and joint infection and one subcutaneous tuberculosis in the RESEARCh, and two urinary infections, four sepses, one infectious gastroenteritis, and one infection not otherwise specified in the REAL. The IR for SAEs

Table 2. Reasons for drug discontinuation of patients with RA treated with ETN or ADA in Korean (RESEARCh) and Japanese (REAL) registries.*

| Reasons for drug discontinuation | RESEARCh ($n = 124$)† | REAL ($n = 144$)† |
|---|---|---|
| Adverse events, n (%) | 41 (33.1) | 56 (38.9) |
| Infection, n (%) | 11 (8.9) | 19 (13.2) |
| Pulmonary disease except infection‡, n (%) | 4 (3.2) | 6 (4.2) |
| Allergy reaction, n (%) | 10 (8.1) | 12 (8.3) |
| Malignancy, n (%) | 0 (0) | 4 (2.8) |
| Cardiovascular system disease, n (%) | 2 (1.6) | 3 (2.1) |
| Others, n (%) | 14 (11.3) | 12 (8.3) |
| Lack of efficacy, n (%) | 31 (25.0) | 53 (36.8) |
| Miscellaneous§, n (%) | 52 (41.9) | 35 (24.3) |

Chi-square test was applied to assess differences in the proportion of causes for discontinuation (i.e., adverse event, lack of efficacy, and miscellaneous), and the adjusted residuals were calculated. A significant difference among the two groups ($p = 0.007$) was observed. The adjusted residuals indicated that significantly higher percentage of patients in the REAL stopped the treatment due to lack of efficacy compared to the RESEARCh and significantly more patients in the RESEARCh stopped the treatment due to miscellaneous.

*Values are the number (percentage) of patients who discontinued ETN or ADA because of each reason.

†Number of patients who discontinued ETN or ADA for any reason.

‡Pulmonary diseases except for infection included interstitial pneumonia and other pulmonary diseases.

§Miscellaneous included good control, patients' preference, financial reasons, and pregnancy. Among 52 cases in the RESEARCh, 14 cases discontinued for financial reasons, 6 cases for patients' refusal, 7 cases for procedure, 5 cases for good control, 4 cases for transfer to local clinic, 1 case for pregnancy, 15 cases for other reasons. In the REAL, among 35 cases, 20 cases for good control, 10 cases for patients' preferences, and 5 cases for financial reasons.

Table 3. Occurrence of SAEs in patients with RA treated with ETN or ADA in Korean (RESEARCh) and Japanese (REAL) registries.*

| | RESEARCh 505.2 PY IR (/100 PY) | REAL 762.0 PY IR (/100 PY) | REAL vs. RESEARCh Crude IRR (95% CI) |
|---|---|---|---|
| Total SAEs† | 6.73 (4.74–9.29) | 13.65 (11.21–16.47) | 2.03 (1.38–2.99) |
| Blood and lymphatic system disorders | 0 | 0.52 (0.18–1.25) | NA |
| Cardiac disorders | 0 | 0.66 (0.25–1.44) | NA |
| Endocrine disorders | 0 | 0.26 (0.05–0.84) | NA |
| Eye disorders | 0 | 0.13 (0.01–0.61) | NA |
| Gastrointestinal disorders | 0 | 0.79 (0.33–1.62) | NA |
| General disorder and administration site conditions | 0.20 (0.02–0.92) | 0.39 (0.11–1.05) | 1.99 (0.21–19.12) |
| Hepatobiliary disorders | 0.79 (0.26–1.88) | 0.26 (0.05–0.84) | 0.33 (0.06–1.81) |
| Infections and infestations | 2.97 (1.73–4.77) | 4.99 (3.58–6.77) | 1.68 (0.92–3.05) |
| Injury, poisoning, and procedural complications | 0.99 (0.38–2.17) | 1.31 (0.67–2.33) | 1.33 (0.45–3.88) |
| Investigations | 0 | 0.39 (0.11–1.05) | NA |
| Musculoskeletal and connective tissue disorders | 0.40 (0.08–1.27) | 0.79 (0.33–1.62) | 1.99 (0.40–9.85) |
| Nervous system disorders | 0.20 (0.02–0.92) | 0.39 (0.11–1.05) | 1.99 (0.21–19.12) |
| Renal and urinary disorders | 0 | 0.39 (0.11–1.05) | NA |
| Reproductive system and breast disorders | 0 | 0.26 (0.05–0.84) | NA |
| Respiratory, thoracic and mediastinal disorders | 0.99 (0.38–2.17) | 1.71 (0.96–2.83) | 1.44 (0.55–3.78) |
| Skin and subcutaneous tissue disorders | 0.20 (0.02–0.92) | 0.26 (0.05–0.84) | 1.33 (0.12–14.62) |
| Vascular disorders | 0 | 0.13 (0.01–0.61) | NA |

PY, patient-year; IR, incidence rate; IRR, incidence rate ratio; CI, confidence interval; NA, not applicable
*Crude IR per 100 PY and crude IRR with their 95% CI were calculated for each category of SAEs occurring from the first to the last dose of ETN or ADA.
†SAEs were classified using the SOC of the MedDRA version 11.1.

are summarized in Table 3. The crude IRR comparing the REAL with the RESEARCh for all SAEs was 2.03 (95% CI, 1.38–2.99). The IRR for infections and respiratory diseases were 1.68 (95% CI, 0.92–3.05) and 1.44 (95% CI, 0.55–3.78), respectively (Table 3).

### Factors influencing development of SAEs

To determine factors influencing development of SAEs, we compared patients who had and had not experienced SAEs using a univariate analysis and selected variables with $p$ value $< 0.05$ or those with medical importance for the multivariate analysis. In the RESEARCh, age per decade (hazard ratio [HR] 1.45, 95% CI 1.10–1.91) was identified as the only risk factor for development of SAEs using the multivariate Cox regression model. In the REAL, age per decade (HR 1.54, 95% CI 1.22–1.93), previous use of nonbiologic DMARDs ≥ 4 (HR 1.93, 95% CI 1.20–3.10), concomitant use of oral CSs (PSL-equivalent dose) ≥ 5 mg/day (HR 2.20, 95% CI 1.11–4.35) were identified as risk factors for SAEs using the multivariate Cox regression model. We then combined the patients from the two registries and performed the multivariate Cox regression analysis. In this analysis, the risk for SAEs was significantly higher in older patients (HR 1.47 per decade, 95% CI 1.23–1.74), and with previous use of nonbiologic DMARDs ≥ 4 (HR 1.64, 95% CI 1.09–2.47) and concomitant use of oral CSs (0 < PSL-equivalent dosage < 5 mg/day; HR 1.91, 95% CI 1.04–3.49, ≥ 5 mg/day; HR 2.04, 95% CI 1.18–3.53) (Table 4).

## Discussion

This is the first study to directly compare safety of biologic DMARDs using harmonized methods between two registries from two

Table 4. Factors influencing development of SAEs in patients with RA treated with ETN or ADA in Korean (RESEARCh) and Japanese (REAL) registries.*

| | RESEARCh | | REAL | | Data combined | |
|---|---|---|---|---|---|---|
| Variables at baseline | Univariate | Multivariate | Univariate | Multivariate | Univariate | Multivariate |
| Age by decade | 1.48 (1.15–1.89) | 1.45 (1.10–1.91) | 1.57 (1.27–1.95) | 1.54 (1.22–1.93) | 1.52 (1.31–1.77) | 1.47 (1.23–1.74) |
| Gender (female) | 0.77 (0.33–1.76) | 0.75 (0.32–1.76) | 0.67 (0.40–1.13) | 0.87 (0.51–1.49) | 0.68 (0.44–1.05) | 0.82 (0.52–1.28) |
| Previous nonbiological DMARDs ≥ 4 | 1.50 (0.72–3.14) | 1.14 (0.53–2.43) | 2.20 (1.39–3.47) | 1.93 (1.20–3.10) | 1.64 (1.12–2.40) | 1.64 (1.09–2.47) |
| Concomitant use of MTX | | | | | | |
| 0 mg/week | 1 | 1 | 1 | 1 | 1 | 1 |
| 0 < MTX < median value† (mg/week) | 0.42 (0.18–1.01) | 0.40 (0.17–0.97) | 0.70 (0.37–1.32) | 0.90 (0.46–1.76) | 0.60 (0.38–0.95) | 0.78 (0.48–1.27) |
| MTX ≥ median value (mg/week) | 0.46 (0.21–0.99) | 0.49 (0.22–1.08) | 0.56 (0.32–0.97) | 1.00 (0.55–1.83) | 0.44 (0.27–0.72) | 0.67 (0.37–1.21) |
| Concomitant use of corticosteroid | | | | | | |
| 0 mg/day | 1 | 1 | 1 | 1 | 1 | 1 |
| 0 < PSL < 5 mg/day | 1.94 (0.76–4.96) | 2.01 (0.78–5.20) | 2.20 (1.00–4.84) | 1.85 (0.82–4.17) | 1.98 (1.09–3.60) | 1.91 (1.04–3.49) |
| ≥ 5 mg/day | 1.77 (0.66–4.71) | 1.60 (0.60–4.33) | 2.66 (1.38–5.11) | 2.20 (1.11–4.35) | 2.47 (1.44–4.23) | 2.04 (1.18–3.53) |
| Chronic pulmonary disease‡ | 1.84 (0.56–6.03) | 1.17 (0.35–3.98) | 2.51 (1.56–4.04) | 1.66 (0.97–2.83) | 2.58 (1.69–3.93) | 1.56 (0.97–2.50) |
| Chronic renal disease§ | 0.53 (0.07–3.89) | 0.35 (0.05–2.61) | 2.33 (0.85–6.39) | 1.55 (0.55–4.37) | 1.29 (0.53–3.17) | 0.91 (0.36–2.27) |
| Nationality (Japan) | | | | | 1.52 (1.01–2.29) | 0.95 (0.55–1.64) |

DMARD, disease-modifying antirheumatic drug; MTX, methotrexate; PSL, prednisolone
*Cox regression model analysis, adjusted for the variables included in the table.
†Median value of each registry; 15 mg/week for the RESEARCh, 8 mg/week in the REAL, 10 mg/week in the data combined.
‡Chronic pulmonary disease included interstitial lung disease, chronic obstructive pulmonary disease, and asthma.
§Chronic renal disease means chronic kidney stages 3, 4, or 5.