JAK2-STAT3 Blockade by AG490 Suppresses Autoimmune Arthritis in Mice via Reciprocal Regulation of Regulatory T Cells and Th17 Cells

Jin-Sil Park, Jennifer Lee, Mi-Ae Lim, Eun-Kyung Kim, Sung-Min Kim, Jun-Geol Ryu, Jae Ho Lee, Seung-Ki Kwok, Kyung-Su Park, Ho-Youn Kim, Sung-Hwan Park and Mi-La Cho

*J Immunol* 2014; 192:4417-4424; Prepublished online 31 March 2014; doi: 10.4049/jimmunol.1300514

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JAK2-STAT3 Blockade by AG490 Suppresses Autoimmune Arthritis in Mice via Reciprocal Regulation of Regulatory T Cells and Th17 Cells


IL-6-mediated STAT3 signaling is essential for Th17 differentiation and plays a central role in the pathogenesis of rheumatoid arthritis. To investigate the molecular mechanism underlying the antirheumatic effects and T cell regulatory effects of STAT3 inhibition, we studied the effects of the JAK 2 inhibitor AG490 on Th17 cell/regulatory T cell (Treg) balance and osteolastogenesis. AG490 was administered to mice with collagen-induced arthritis (CIA) via i.p. injection, and its in vivo effects were determined. Differential expression of proinflammatory cytokines, including IL-17A, IL-1β, and IL-6, was analyzed by immunohistochemistry. Levels of phosphorylated STAT3 and STAT5 and differentiation of Th17 cells and Tregs after AG490 treatment in our CIA model were analyzed by immunostaining. In vitro development of Th17 cells and Tregs was analyzed by flow cytometry and real-time PCR. AG490 ameliorated the arthritic phenotype in CIA and increased the proportion of Foxp3+ Tregs. In contrast, the proportion of IL-17A–producing T cells and levels of inflammatory markers were reduced in AG490-treated mice. Numbers of p-STAT3+ CD4+ T cells and p-STAT5+ CD4+ T cells were reduced and elevated, respectively, after treatment with AG490. Furthermore, AG490 markedly increased the expression of molecules associated with Treg development (ICOS, programmed cell death protein 1, ICAM-1, and CD103). The development and function of osteoclasts were suppressed by AG490 treatment. Our results suggest that AG490, specifically regulating the JAK2/STAT3 pathway, may be a promising treatment for rheumatoid arthritis. The Journal of Immunology, 2014, 192: 4417–4424.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by the hyperplasia of synovial tissue and progressive destruction of articular cartilage, bone, and ligaments (1–3). The chronic progressive course of RA can result in complete ankylosis and subsequent loss of joint function.

The pathogenesis of RA is a complex process mediated by an interdependent network of cytokines, proteolytic enzymes, and prostanoids. In inflamed RA joints, local production of proinflammatory cytokines, such as IL-1, TNF-α, IL-6, and IL-17, plays an important role (4).

Among various subtypes of Th cells with distinct functions and cytokine profiles, Th17 cells can be distinguished from Th1 cells and Th2 cells in that they produce IL-17A, IL-17F, IL-21, and IL-22 (5). Th17 cells are involved in the pathogenesis of various autoimmune disorders, such as experimental autoimmune encephalomyelitis, RA, and allergic responses (6, 7). In RA, IL-17 stimulates various pathogenic cells by activating proinflammatory mediators, such as IL-1, IL-6, IL-23, and TNF-α (8–10). IL-6 plays a crucial role in the differentiation of naïve T cells into Th17 cells by activating retinoic acid receptor-related orphan nuclear receptor (RORαT) (11), a specific transcriptional regulator, critical for the expression of IL-17 (6, 12, 13). Engagement by IL-6 of membrane gp130, a common receptor chain for IL-6 and IL-6R, leads to the activation of JAK tyrosine kinases and subsequent phosphorylation of tyrosine residues within the gp130 cytoplasmic tail. Moreover, IL-6–induced JAK family members activate JAK/STAT and MAPK cascades (14). JAK2 activates downstream genes through STAT3 or STAT5 and is responsible for transducing signals for several proinflammatory cytokines involved in the pathogenesis of RA, including IL-6, IFN-γ, and IL-12 (15).

The therapeutic effects of JAK inhibition in RA were reported recently. Tofacitinib (CP-690,550), a pan-JAK inhibitor that is currently in use for the treatment of RA, was shown to suppress the production of IL-17 and IFN-γ, as well as the proliferation of CD4+ T cells, under conditions of anti-CD3 and anti-CD28 stimulation (16), and to decrease T lymphocyte receptor activator for NF-κB ligand (RANKL) production (17). However, pan-JAK
inhibitors may have safety issues linked to “off-target” effects, because JAK proteins are essential in signaling pathways that maintain physiological immune responses and processes, including hematopoiesis (18). To avoid these concerns, the effects of more selective JAK2 inhibitors are gaining attention. JAK2 inhibitors are classic therapeutics for myeloproliferative diseases because mutations of JAK2, which is associated with erythropoietin and tyrosine stromal lymphopoietin receptors, play a critical role (19-21). Recently, the JAK2 inhibitors CEP-33779 and SB1578 were investigated in an RA model (22, 23). AG490 has been widely used as a JAK2 inhibitor in various settings, and it appears to specifically suppress JAK2/STAT3 signaling (24-27). However, its therapeutic potential in autoimmune arthritis has never been addressed. In this study, we demonstrated that AG490 attenuated the incidence and severity of collagen-induced arthritis (CIA), through the reciprocal regulation of Th17 cells/regulatory T cells (Tregs), and suppressed osteoclast differentiation.

MATERIALS AND METHODS

Induction of CIA and treatment with AG490

Type II collagen (CII) was dissolved overnight in 0.1 N acetic acid (4 mg/ml) with gentle rotation at 4°C. Eight-week-old male DBA/1J mice were injected intradermally at the base of the tail with 100 μg CII emulsified in CFA. Two weeks later, they were boosted intradermally with 100 μg CII in IFA. To assess the influence of AG490 on symptom severity in the CIA model, mice were treated with AG490 (500 μg/mouse) in saline or with vehicle alone by i.p. injection three times a week, from day 2 after a booster immunization, for 3 wk. To evaluate the therapeutic effects of AG490, mice were injected with AG490 (500 μg/mouse) in saline or with vehicle from day 28 after the first immunization, when clinically evident clinical arthritis was determined by three independent observers. The mice were observed twice a week for the onset and severity of joint inflammation for up to 8 wk after the primary immunization. The severity of arthritis was assessed on a scale of 0–4, as described previously (28): 0 = no edema or swelling, 1 = slight edema and erythema limited to the foot or ankle, 2 = slight edema and erythema from the ankle to the tarsal bone, 3 = moderate edema and erythema from the ankle to the tarsal bone, and 4 = edema and erythema from the ankle to the entire leg. The arthritis score for each mouse was expressed as the sum of the scores of four limbs, with a maximum possible score of 12. The mean arthritis index was used to compare the control and experimental groups.

Clinical assessment of arthritis

Histology

Mouse joint tissues were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifier, embedded in paraffin, and sectioned. The sections were stained with H&E, safranin O, and toluidine blue to detect proteoglycans.

Immunohistochemistry

Mouse joint tissues were fixed in 10% formalin, decalcified in Calci-Clear Rapid bone decalcifier, embedded in paraffin, and sectioned (29). The sections were deparaffinized using xylene and dehydrated in a gradient of alcohol solutions. Endogenous peroxidase activity was quenched with 3% H2O2 in methanol. Immunohistochemistry was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). Tissues were incubated first with primary Abs against RANK, IL-17A, IL-6 (Abcam), IL-1β, TNF-α, and vascular endothelial growth factor (VEGF) A and an isotype control (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Then the tissues were incubated with a biotinylated secondary Ab and a streptavidin-peroxidase complex for 1 h. The final colored product was developed using DAB Chromogen (Thermo). Finally, the sections were counterstained with hematoxylin and photographed using a photomicroscope (Olympus, Tokyo, Japan).

Ag-specific T cell proliferation

Single-cell suspensions prepared from the spleens or lymph nodes were cultured at 2 × 10^3 cells/well in 96-well plates with CII (100 μg/ml). Cultured cells were pulsed with 0.5 mCi [3H]thymidine (GE Healthcare, Little Chalfont, U.K.) in 20 μl RPMI 1640 for during the last 16 h of incubation. The incorporation of [3H]thymidine was determined using a Betaplate scintillation counter (PerkinElmer, Wellesley, MA).

Measurement of CII-specific Abs

Blood was drawn from the orbital sinuses of AG490- and vehicle-treated mice, and sera were stored at −20°C until use. Microtiter plates were coated with CII (4 μg/ml in PBS) at 4°C overnight, followed by a blocking step for 30 min at room temperature. Serum samples were diluted 1:10,000 in TBS (pH 8) containing 1% BSA and 0.5% Tween-20 and incubated in the microwell plates for 1 h, after which the plates were washed five times. The concentrations of CII-specific IgG and IgG2a were measured using mouse IgG1FgG2a ELISA Quantitation Kits (Bethyl Laboratories, Montgomery, TX), respectively. The absorbance values were determined with an ELISA microplate reader operating at 450 nm.

ELISA

The amounts of IL-17A and TNF-α in culture supernatants were measured by sandwich ELISA (R&D Systems). Absorbance at 405 nm was measured using an ELISA microplate reader.

CD4+ T cell purification and stimulation

To purify splenic CD4+ T cells, the splenocytes of DBA/1J mice were incubated with CD4-coated magnetic beads and isolated using MACS separation columns (Miltenyi Biotec). To establish Th17 cell–polarizing conditions, the MACS-selected Th17+ T cells were stimulated with plate-bound anti-CD3 (0.5 μg/ml), anti-CD28 (1 μg/ml), anti-IFN-γ (2 μg/ml), anti-IL-4 (2 μg/ml), TGF-β (20 ng/ml), and IL-6 (20 ng/ml) for 72 h. Total RNA was extracted using TRIzol reagent. The supernatants were assayed for IL-17A and TNF-α levels.

Flow cytometry

For intracellular cytokine staining in mice, cells were stimulated with 25 ng/ml PMA and 250 ng/ml ionomycin in the presence of GolgiStop for 4 h. The following Abs were used for intracellular staining: anti-IL-17A–FITC, anti-IL-17A–PE, anti-Foxp3–FITC, anti-Foxp3–PE, anti-ICOS–FITC, anti-CD69–FITC, anti-CD127–FITC, and anti-CD123–FITC (all eBioscience). Events were recorded and analyzed with FlowJo software (TreeStar, Ashland, OR).

Confocal microscopy of immunostaining

Spleen tissue sections were snap-frozen in liquid nitrogen and stored at −70°C. Spleen tissue sections (7 μm) were fixed in acetone and stained for Tregs using PE-labeled anti-CD3, PerCP-labeled anti-CD4, and APC-labeled anti-CD25. The CII–specific IgG Abs were detected using FITC-labeled anti-G2b12 IgG2a murine Abs (CII-specific). Tregs were stained with FITC-labeled anti–CD25, PE-labeled anti–CD4, CyChrome-labeled anti–CD28, and APC-labeled anti–CD127. Stained sections were analyzed using an LSM 510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany). Positive cells were counted at higher magnification by four individuals.

Immunoblot analysis

Cells were lysed in Halt protein lysis buffer containing Halt phosphatase inhibitor (Therm Pierce). Lysates were centrifuged for 15 min at 14,000 × g at 4°C, and protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Lysates were separated by SDS-PAGE and transferred to a Hybond ECL membrane (GE Healthcare) for Western blot analysis using the SNAP i.d. Protein Detection System (Millipore, Billerica, MA). The following Abs were used: anti–β-actin (Sigma, St. Louis, MO). After washing, HRP-conjugated secondary Abs were added. Hybridized bands were detected using an ECL detection kit (Pierce, Rockford, IL) and Hyperfilm (Agfa, Belgium).

Mouse in vitro osteoclastogenesis

Isolation of bone marrow–derived monocytes/macrophages (BMMs) and differentiation of osteoclast precursor cells (preosteoclasts) were performed as described (30). Three days later, nonadherent cells were washed out, and preosteoclasts were cultured in the presence of 10 ng/ml M-CSF, 50 ng/ml RANKL (PeproTech, London, U.K.), and various concentrations of AG490 for 4 d to generate osteoclasts.

Tartrate-resistant acid phosphatase staining

A commercial TRAP kit (Sigma) was used, according to the manufacturer’s instructions, with omission of hematoxylin counterstaining. Tartrate-resistant

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acid phosphatase (TRAP) cells containing three or more nuclei were scored as osteoclasts.

Bone resorption

Mouse BMMs, prepared using the method described above, were cultured in dentine discs (Immunodiagnostic Systems, Boldon, U.K.). The erosive areas were identified using Tomoro analySIS TS Lite software (Olympus, Münster, Germany).

Real-time PCR

A LightCycler 2.0 instrument (Roche Diagnostics) and software (version 4.0) were used for PCR amplification and analysis. All reactions were performed with LightCycler FastStart DNA Master SYBR Green I, according to the manufacturer’s instructions. The following primers were used for mouse sequences: IL-17A, 5'-CCTCAAGGCTCAGGTGTC-3' (sense) and 5'-GAGGTCATTTTGGCAGCA-3' (antisense); Foxp3, 5'-GGGCTTCTCAGAGGAGCAG-3' (sense) and 5'-GCTGATCATGTTCCCTGTT-3' (antisense); RORγt, 5'-GTGCTGGGCTACCCTACTG-3' (sense) and 5'-GGCCCTTCTTGAGTGACC-3' (antisense); IFN regulatory factor 4 (IRF4), 5'-GAACAGCTCCAGTCCCATACCC-3' (sense) and 5'-GAGGGTCGGTAATCCT-3' (antisense); integrin-β, 5'-CTGTCCAGACCAAGGGTACAGCCT-3' (sense) and 5'-ACATAGCCCACACCGTTCTC-3' (antisense); and β-actin, 5'-ACTG-3' (antisense) and 5'-CTGCGCTG-3' (antisense). The level of mRNA expression was normalized to that of β-actin mRNA.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 4 for Windows; GraphPad, San Diego, CA). When comparing pairs of groups, the Mann-Whitney U test was used for continuous variables, and the χ² test was used for categorical variables. For multiple comparisons, ANOVA was used with the Bonferroni post hoc analysis. The p values < 0.05 were considered significant.

Results

AG490 ameliorated the severity of CIA

To assess the in vivo effect of AG490 on autoimmune arthritis, we investigated whether AG490 would suppress rheumatoid inflammation and joint destruction in CIA mice. CIA mice injected with AG490 showed significantly lower arthritic scores and arthritis incidence compared with CIA mice injected with vehicle (Fig. 1A).

![Figure 1](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 1.** Treatment with AG490 reduced the severity of CIA. To assess the influence of AG490 on symptom severity in the CIA model, DBA/1J mice were treated with AG490 (500 μg/mouse) in saline or with vehicle alone via i.p. injection three times a week from day 2 after booster immunization for 3 wk (n = 4/group). (A) Clinical scores and incidence in AG490-treated mice and vehicle-treated mice during the experimental periods. (B) Mice were sacrificed 40 d after the first immunization, and concentrations of CII-specific IgG and IgG2a in sera were determined by ELISA. (C) Proliferation was determined by [3H]thymidine incorporation. Thymidine was added to cultures for the final 16 h before harvesting. (D) Representative histological features of the joints of AG490- or vehicle-treated mice (n = 4 joints/group). H&E, safranin O, and toluidine blue staining and immunohistochemical analysis of TNF-α, VEGF, and RANK. (E) Tissue sections of joints from vehicle- or AG490-treated mice were stained with an anti-TRAP Ab. (F-H) To evaluate the therapeutic effects of AG490, DBA/1J mice were injected with AG490 in saline or with vehicle alone via i.p. injection, three times a week from day 28 after the first immunization, for 3 wk (n = 4/group). (F) Arthritis development was assessed. (G) Mice were sacrificed 70 d after CIA induction, and isolated splenocytes were stimulated in vitro with CII (50 μg/ml). (H) The serum concentration of CII-specific IgG and IgG2a, as well as incorporation of [3H]thymidine after 3 d, was measured. Data are representative of two experiments (mean and SD). *p < 0.05, **p < 0.01, ***p < 0.001.
Serum levels of CII-specific IgG and IgG2a, as well as proliferation of lymphocytes, were significantly lower in AG490-treated mice than in vehicle-treated mice (Fig. 1B, 1C). Histological sections of ankle joints stained with H&E, toluidine blue, and safranin O showed that AG490-treated mice had less severe cartilage loss than did vehicle-treated CIA mice. Also, fewer TNF-α⁺, VEGF⁺, and RANK⁺ cells were observed in the joints of AG490-treated mice (Fig. 1D). To assess the in vivo effects of AG490 on joint destruction by osteoclasts, joint sections from vehicle- and AG490-treated mice were stained to determine TRAP expression. The numbers of TRAP⁺ cells were reduced in the joints of AG490-treated mice (Fig. 1E). Next, we investigated whether AG490 also had therapeutic effect in established arthritis. AG490 was administered from day 28 after the first immunization, when arthritis began to be observed. As in the preventive model, AG490-treated mice exhibited less severe arthritis (Fig. 1F). On day 70 after the first immunization, there was a significant reduction in CII-specific IgG and IgG2a production in AG490-treated mice (Fig. 1G). CII-specific T cell proliferation decreased in response to AG490 (Fig. 1H).

**AG490 regulated in vivo Th17 cells and Tregs in autoimmune arthritis**

Histological sections of the tibiotalar joint of the ankle from CIA mice were stained for several inflammatory cytokines. Fewer IL-17A⁺, IL-1β⁺, and IL-6⁺ cells were observed in the joints of AG490-treated mice than in those of vehicle-treated mice (Fig. 2A). Next, we examined whether JAK2 inhibition was associated with the frequency of Th17 cells and Tregs in vivo. We counted CD4⁺IL-17A⁺ Th17 cells and CD4⁺CD25⁺Foxp3⁺ Tregs in spleens from mice treated with AG490 or vehicle by immunostaining. The number of Th17 cells decreased in response to AG490 treatment, whereas the number of Tregs increased (Fig. 2B, 2C). Because the reciprocal regulation of Th17 cells and Tregs is

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**FIGURE 2.** JAK2 inhibition regulated inflammation-related factors in vivo. (A) Representative histological features of the tibiotalar joints of saline-treated CIA mice and AG490-treated mice. Immunohistochemical staining for IL-17A, IL-1β, and IL-6. (B) Spleens were subjected to immuno-staining for CD4⁺IL-17A⁺, CD4⁺p-STAT3 (Tyr705)⁺, and CD4⁺p-STAT3 (Ser727)⁺ cells. Tregs were counted in four independent quadrants. (C) Spleens were subjected to immunostaining for CD4⁺CD25⁺Foxp3⁺ or CD4⁺p-STAT5⁺ cells. Tregs were counted in four independent quadrants. *p < 0.05, **p < 0.01, ***p < 0.001.
dependent on competition between p-STAT3 and p-STAT5, the frequency of p-STAT3\(^{705}\) or p-STAT5\(^{727}\) cells also was assessed. The results showed fewer CD4\(^{+}\)p-STAT3\(^{705}\) or p-STAT5\(^{727}\) T cells. Notably, the number of CD4\(^{+}\)p-STAT5\(^{727}\) T cells was increased by AG490 treatment, although the difference was not statistically significant. This suggests that AG490 specifically inhibits STAT3 phosphorylation, leaving the JAK2/STAT5 pathway intact.

**AG490 suppressed Th17 cell differentiation in vitro**

To examine the inhibitory effects of AG490 on in vitro Th17 cell differentiation, isolated splenic CD4\(^{+}\) T cells were incubated in the presence or absence of AG490 for 2 h and then cultured under Th17-polarizing conditions (anti-CD3, anti-CD28, TGF-\( \beta \), IL-6) for 72 h. The suppression of JAK2 by AG490 reduced the number of CD4\(^{+}\)IL-17A\(^{+}\) Th17 cells, whereas the number of CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) cells increased in proportion to the concentration of AG490 (Fig. 3A). IL-17A mRNA synthesis and protein levels showed similar decreases, whereas Foxp3 mRNA synthesis was increased under the tested conditions (Fig. 3B). The expression of Th17-related molecules, such as ROR\( \gamma T\), IRF4, CCR6, and Runx1, decreased in response to AG490, although the difference reached statistical significance for CCR6 only (Fig. 3C). To investigate the inhibitory effect of AG490 on STAT3 phosphorylation on the protein level, Western blot analysis was performed using splenic CD4\(^{+}\) T cells that were pretreated with AG490 and cultured under Th17-polarizing conditions for 24 or 48 h. As shown in Fig. 3D, treatment with AG490 suppressed phosphorylation of STAT3 in Tyr\(^{705}\) and Ser\(^{727}\) under these conditions.

**AG490 induced Tregs under inflammatory conditions**

To assess Treg induction by AG490, CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\)ICOS\(^{+}\), CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\)PD-1\(^{+}\), CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\)ICAM-1\(^{+}\), and CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\)CD103\(^{+}\) cells were counted after CD4\(^{+}\) T cells were cultured under Th17-polarizing conditions in the presence or absence of AG490. Flow cytometric analysis showed that in vitro treatment with AG490 upregulated Foxp3, as well as ICOS, PD-1, ICAM-1, and CD103, which are implicated in the differentiation of Tregs (Fig. 4).

**AG490 suppressed the differentiation and function of osteoclasts**

To verify the suppressive effects of AG490 on osteoclast differentiation in vitro, mouse bone marrow cells were differentiated into osteoclasts in the presence of M-CSF, RANKL, and various concentrations of AG490. The concentration used in the experiment did not affect the cell viability, as determined using the CCK8 kit (data not shown). AG490 treatment suppressed osteoclast

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**FIGURE 3.** JAK2 inhibition affected the differentiation of Th17 cells and Tregs in vitro. CD4\(^{+}\) T cells were isolated from the spleens of DBA/1J mice, pretreated with a given concentration of AG490 for 2 h, and incubated for 3 d under Th17-polarizing conditions. (A) Numbers of CD4\(^{+}\)IL-17A\(^{+}\) cells and CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) cells were analyzed by intracellular flow cytometry. Numbers in the plots indicate the percentage of CD4\(^{+}\)IL-17A\(^{+}\) cells or CD4\(^{+}\)CD25\(^{+}\)Foxp3\(^{+}\) cells. (B) Expression of IL-17A and Foxp3 was analyzed by real-time PCR. Concentrations of IL-17A protein in culture supernatants were measured by ELISA. (C) Expression of ROR\( \gamma T\), IRF4, CCR6, and Runx1 was analyzed by real-time PCR. (D) Splenic CD4\(^{+}\) T cells were isolated from DBA/1J mice, pretreated with AG490 for 2 h, and cultured under Th17-polarizing conditions for 24 or 48 h. Protein extracts from these cells were analyzed by immune blotting using Ab to p-STAT3 (Tyr\(^{705}\), Ser\(^{727}\)) as probes. \(*p < 0.05, **p < 0.01, ***p < 0.001.\)
differentiation in vitro in a dose-dependent manner (Fig. 5A). The functional activity of osteoclasts was assessed using dentine slices. The resorbed area was smaller with AG490 treatment (Fig. 5A). To determine whether AG490 regulated the mRNA levels of molecules known to be strongly expressed in osteoclasts, the relative mRNA expression levels of TRAP, integrin-β3, cathepsin K, and MMP-9 were measured by quantitative real-time PCR. All decreased in response to AG490 (Fig. 5B).

Discussion
Given the great success using biologics targeting inflammatory cytokines, such as TNF-α and IL-6, in the treatment of RA, small molecules involved in the signal-transduction pathways of these inflammatory cytokines have become attractive targets. Among the various pathways identified, JAK/STAT has received particular attention. This is a common signaling pathway of classical immune (type I and type II) cytokines, whose receptors are deprived
of intrinsic protein kinase activity and require kinases to activate downstream-signaling cascades (31).

In the current study, we demonstrated that the JAK2 inhibitor AG490 suppressed Th17 cell differentiation by inhibiting STAT3 activation. AG490 also increased STAT5 phosphorylation, thereby increasing the number of Tregs. Moreover, it showed a direct inhibitory effect on osteoclastogenesis.

Given that IL-6–mediated JAK/STAT3 activation is crucial in the development of pathogenic Th17 cells, inhibitors of JAK/STAT signaling can block this major pathogenic process and are promising therapeutics in RA. Notably, tofacitinib (a JAK1 and JAK3 inhibitor) and baricitinib (a JAK1 and JAK2 inhibitor) have shown clinical efficacy in RA. The exact role of JAK inhibition in the pathogenesis of RA is beginning to be uncovered. Yarilina et al. (32) demonstrated that tofacitinib suppressed macrophage activation and attenuated TNF responses in a serum-transfer arthritis model. Maeshima et al. (16) suggested that the therapeutic effect of tofacitinib results from inhibition of IFN-γ and IL-17A. LaBranche et al. (17) reported that tofacitinib suppressed arthritic joint structural damage by decreasing RANKL production.

Because IL-6R uses JAK1 and JAK2 to activate downstream signaling, it may be inferred that AG490 would have a therapeutic effect on RA. Moreover, AG490 was reported to inhibit IL-6 signaling by downregulating gp130 expression, independent of JAK/STAT signaling (33). Indeed, the frequency of Th17 cells was reduced in AG490-treated CIA mice. This change was associated with a reduction in the number of p-STAT3+ CD4+ T cells. These findings are in agreement with a previous study (34) that revealed that the transcription of IL-17A is regulated by direct, reciprocal actions of STAT3 and STAT5, which compete for binding to the same region. It is not clearly understood why STAT3 inhibition leads to enhanced phosphorylation of STAT5. However, the results of our previous and current study consistently demonstrated the reciprocal activation of the two molecules (35–37). Because the reciprocal regulation of STAT3 and STAT5 was observed during an inflammatory condition (in the CIA model or under Th17-differentiating conditions), it appears that the T cells cannot be differentiated into Th17 cells due to the lack of p-STAT3 whereas they can be converted to Treg due to the activated p-STAT5 in the presence of AG490. This explanation also reflects the concept of the plasticity among Th17 cells and Tregs in their differentiation (38). In addition, we recently reported that modulation of STAT3 by small interfering RNA results in the reduction of Th17 differentiation, as well as a reciprocal increase in Tregs in patients with RA (39). In the current study, the number of Tregs also was elevated after STAT3 inhibition. Therefore, it appears that, similar to small interfering RNA, AG490 specifically inhibits STAT3 and shows therapeutic potential in a CIA model.

To confirm the effect of AG490 on Treg differentiation under inflammatory conditions, CD4+ T cells were cultured under Th17-polarizing conditions. AG490 increased the frequency of CD25+Foxp3+ cells in a dose-dependent manner. This was consistent with a previous study (24) that showed that AG490 treatment increased the number of CD4+CD25+Foxp3+ cells in NOD mice. In addition to Foxp3, molecules associated with Treg development were upregulated by AG490 treatment. ICOS and PD-1 are required for the development of Tregs and their regulation of effector T cells (40, 41). ICAM-1 is associated with TGF-β-induced Foxp3 expression (42), and CD103 has been used as a marker of in vivo–activated CD4+Foxp3+ Tregs (43). The upregulation of these molecules further supports the notion that Treg development is enhanced by AG490 treatment.

We also demonstrated that osteoclastogenesis in murine BMMs was inhibited by AG490 treatment and that the mRNA expression of molecules associated with osteoclast activity was reduced. The role of JAK2/STAT3 in osteoclastogenesis has not been elucidated. It was recently reported that RANKL, a key factor in osteoclastogenesis, induces phosphorylation of STAT3 at Ser727 and that this is critical for the RANKL-mediated reduction in CXCR6 expression (44). That study showed that treatment with AG490 inhibited STAT3 activation and did not result in a reduction in CXCR6 during osteoclastogenesis. Recently, we demonstrated that several STAT3 inhibitors, including STA-21 (36) and GRIM-19 (45), suppressed osteoclastogenesis both in vivo and in vitro. Together with the results of the present study, this suggests that STAT3 may have an important role in osteoclastogenesis. Nevertheless, another JAK inhibitor, tofacitinib, does not have a direct effect on osteoclastogenesis (17); instead, it suppresses osteoclastogenesis by inhibiting RANKL production by human T lymphocytes. Whether this discrepancy is due to the difference between the JAK subtypes that each drug inhibits or the unique properties of the drug itself requires further investigation.

Because JAK signaling is essential in hematopoiesis, it should be clarified that the dose used to inhibit arthritis does not affect normal erythropoiesis, to confer a novel meaning to our data. Indeed, when we compared the level of hemoglobin between vehicle-treated and AG490-treated CIA mice after 3 wk of treatment, there was no significant difference between the two groups (data not shown), relieving concerns about ineffective hematopoiesis with AG490 treatment.

In conclusion, the JAK2 inhibitor AG490 ameliorated CIA by suppressing Th17 and through reciprocal upregulation of Tregs, as reflected by patterns of STAT3/STAT5 phosphorylation and the expression of molecules associated with Th17 cells and Tregs. It further inhibited the development and function of osteoclasts. Our results suggest that AG490, which specifically regulates the JAK2/STAT3 pathway, may be a promising treatment for RA.

Acknowledgments
We thank the Department of Laboratory Animal, The Catholic University of Korea, for outstanding animal husbandry.

Disclosures
The authors have no financial conflicts of interest.

References


