Retinoic Acid Attenuates Rheumatoid Inflammation in Mice

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*J Immunol* 2012; 189:1062-1071; Prepublished online 13 June 2012;
doi: 10.4049/jimmunol.1102706
http://www.jimmunol.org/content/189/2/1062

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The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Retinoic Acid Attenuates Rheumatoid Inflammation in Mice

Seung-Ki Kwok,*1 Mi-Kyung Park,†,1 Mi-La Cho,‡,2 Hye-Jwa Oh,† Eun-Mi Park,† Dong-Gun Lee,‡ Jennifer Lee,§ Ho-Youm Kim,*† and Sung-Hwan Park*,2

Retinoic acid is the active vitamin A derivative and is well-known to have diverse immunomodulatory actions. In this study, we investigated the impact of all-trans retinoic acid (ATRA), a biologic key metabolite of vitamin A, on the development of arthritis and the pathophysiologic mechanisms by which ATRA might have antiarthritic effects in animal model of rheumatoid arthritis (RA; collagen-induced arthritis [CIA] in DBA/1J mice). We showed that treatment with ATRA markedly suppressed the clinical and histologic signs of arthritis in the CIA mice. It reduced the expression of IL-17 in the arthritic joints. Interestingly, Foxp3+ regulatory T cells were markedly increased and IL-17–producing CD4+ T cells (Th17 cells) were decreased in the spleens of ATRA-treated mice. In vitro treatment with ATRA induced the expression of Foxp3 and repressed the IL-17 expression in the CD4+ T cells in mice. ATRA suppressed the production of total IgG and IgG2a in splenocytes that were stimulated by LPS. It also reduced serum levels of total IgG and IgG2a anti-collagen Abs and germinal center formation in CIA mice. In addition, the ATRA-treated mice showed decreased osteoclast formation in arthritic joints. Moreover, ATRA downregulated the expression of receptor activator of NF-κB ligand, the leading player of osteoclastogenesis, in the CD4+ T cells and fibroblast-like synoviocytes from patients with RA. Furthermore, ATRA prevented both human monocytes and mice bone marrow-derived monocytes/macrophage cells from differentiating into osteoclasts. These data suggest ATRA might be an effective treatment modality for RA patients. The Journal of Immunology, 2012, 189: 1062–1071.

Rheumatoid arthritis (RA) is a multisystemic autoimmune disease of unknown cause, and RA is characterized by hyperplastic synovial membrane that is capable of destroying the adjacent articular cartilage and bone (1, 2). Although the pathogenesis of RA has not been completely elucidated, a variety of cytokines such as TNF-α and IL-6 play major roles in the development of RA. It is well-known that diverse biologic agents targeting proinflammatory cytokines like TNF-α and IL-6 have proved to be significantly superior to conventional disease-modifying antirheumatic drugs (DMARDs) (3–6). However, some populations of RA patients are still refractory to various biologic agents, as well as DMARDs. Therefore, the development of new therapeutic strategies is mandatory.

Retinoic acid is an acidic form of vitamin A, and it is wellknown to play a major role in a wide range of physiologic pathways such as cell proliferation, embryogenesis, differentiation, morphogenesis, and inflammation (7). It has antitumor and diverse immunomodulatory actions (8), and it has been used for the treatment of acute promyelocytic leukemia (9) and inflammatory skin diseases like acne (10) and psoriasis (11). Retinoic acid also has been demonstrated to regulate reciprocal differentiation between regulatory T cells and Th17 cells. It can promote anti-inflammatory Foxp3-expressing regulatory T cells and suppress proinflammatory Th17 cells (12, 13). Regarding the effect of retinoic acid on autoimmune diseases, a few reports have shown the therapeutic efficacy of retinoic acid in animal models of multiple sclerosis (14) and systemic lupus erythematosus (15, 16). As for the role of retinoic acid in RA, which is the prototype of autoimmune diseases, a few investigations have shown that retinoic acid had a therapeutic impact on animal models of RA (17-20). However, the precise mechanism of the therapeutic effect mediated by retinoic acid in rheumatoid inflammation still remains to be determined.

Therefore, we hypothesized that all-trans retinoic acid (ATRA), which is a biologically active, key metabolite of vitamin A, would suppress arthritis in an animal model of RA, that is, collagen-induced arthritis (CIA). To elucidate the possible mechanism by which ATRA has a therapeutic effect, we investigated whether ATRA affects Th17 cells, which are a proinflammatory Th cell subset that has been deeply implicated in the development of RA (21), and Foxp3-expressing regulatory T cells, which suppress autoimmune processes and maintain peripheral tolerance (22). We also examined the effect of ATRA on humoral immune responses by evaluating Ab production and germinal center (GC) formation. We finally investigated the impact of ATRA on osteoclasts, which are critical cells that have been deeply implicated in bone destruction in RA, in vivo and in vitro.
Materials and Methods

Animals

Male DBA/1J mice were purchased from the Charles River Laboratory (Yokohama, Japan). The mice were fed food and water ad libitum, and they were allowed to acclimatize themselves for 2 wk before the initiation of experiment. The animals were kept under specific pathogen-free conditions and were studied at 7–10 wk of age. All the experimental procedures were examined and approved by the Animal Research Ethics Committee at The Catholic University of Korea.

Induction and evaluation of CIA

DBA/1J mice (7 wk of age) were intradermally immunized (day 0) at the base of the tail with 100 μg bovine type II collagen (CII; Chondrex, Redmond, WA) with CFA (Chondrex).

To investigate the effect of ATRA on CIA, we injected DBA/1 mice i.p. three times a week for 9 wk with 0.5 mg/kg ATRA or cottonseed oil (vehicle) as a control, beginning on day 7 after the primary immunization, and the mice were monitored for 10 wk. The mice were visually examined three times per week for the appearance of arthritis in the peripheral joints, and the arthritis score index for the disease severity was as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the midfoot (tarsals) or ankle joint; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and moderate swelling extending from the ankle to the metatarsal joints; 4, erythema and severe swelling encompassing the ankle, foot and digits. The maximum possible score per mouse was 16. The scoring was done by two independent observers who were without knowledge of the experimental and control groups.

Histopathology of arthritis

The mouse joint tissues were fixed in 4% paraformaldehyde, decalcified in EDTA bone decalcifier, and embedded in paraffin. Seven-micrometer tissue sections of the spleens were stained using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The tissues were first incubated with the primary anti-nitrotyrosine, anti-inducible NO synthase (anti-iNOS), anti–TNF-α, anti–IL-1β, anti–IL-17, anti-receptor activator of NF-κB ligand (anti-RANKL), anti-receptor activator of NF-κB (anti-RANK), anti-calcitonin receptor (anti-CTR), anti–NFATc1 Abs (all from Santa Cruz Biotechnology, Santa Cruz, CA); anti–IL-6 (Abcam), anti–IL-21 (R&D Systems), mouse IgG isotype (for FITC conjugates), goat IgG isotype (for PE conjugates), rabbit IgG isotype (for anti-CD3–stimulated CD4+ T cells) and donkey IgG isotype (for anti-CT2 cells) for immunohistochemistry was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were counterstained with hematoxylin. Images were developed using 3,3-diaminobenzidine chromogen (DAKO, Carpinteria, CA). The sections were captured using a DP71 digital camera (Olympus, Center Valley, PA) at 400 magnification. For histologic evaluation of CIA, sections were evaluated in a blind manner, as has been described previously. The scores were evaluated as previously described (23).

Confocal microscope

For confocal staining, 7-μm tissue sections of the spleens were stained using FITC-conjugated anti-Foxp3, PE-conjugated anti–IL-17, aliphosphorycycin-conjugated anti-CD25, aliphosphorycycin-conjugated anti-B220, FITC-conjugated anti–GL-7, and PE-conjugated anti-CD138 (all from eBioscience, San Diego, CA); then the sections were incubated with biotinylated anti-CD4 (BD Pharmingen) and streptavidin Cy-3 (Zymed) overnight at 4°C. The stained sections were analyzed using a Zeiss microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany) at ×400 magnification.

Anti-CT2 Ab measurement

The anti-CII total IgG and IgG2a Abs were measured using the mouse IgGl/IgG2a ELISA quantitation kit (Bethyl Lab, Montgomery, TX). The levels of total IgG and IgG2a were measured in the mice sera that were diluted 25,000-fold. In some experiments, after the spleens were excised, splenocyte suspension was prepared by gently teasing the spleens with forceps. RBCs were lysed by the addition of a lysis buffer (0.16 M ammonium chloride in Tris buffer, pH 7.2) at 37°C for 2 min. The final spleenocyte concentration was adjusted to 5 × 107 cells/well in a 48-well plate and incubated with ARTA for 2 h before 100 ng/ml LPS stimulation at 37°C in a 5% CO2 humidified incubator. After 6 d in culture, the supernatant was collected and frozen at −70°C for the assays of IgG. The OD in each well was measured at a 450-nm wavelength in an ELISA plate reader (Bio-Rad, Hercules, CA).

CII-specific T cell response

The spleen was minced and the cells were filtered through a cell strainer and centrifuged at 1500 rpm at 4°C for 10 min. CD4+ T cells were purified by negative selection using the Untouched CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). CD25+ cells were depleted from isolated CD4+ T cells using CD25 Microbeads (Miltenyi Biotec) to obtain the CD4+CD25− population. All the magnetic separations were performed with the Automacs Separator, following the manufacturer’s instructions. The CD4+CD25+ T cells (1 × 105/well) were cultured with irradiated T cell-depleted splenic APCs (1 × 105/well) in 96-well plates at 37°C for 72 h in the presence of 50 μg/ml CII Ag. The cells were cultured for 72 h, and 18 h before the termination of culture, 1 μCi [3H]thymidine (Amersham Pharmacia Biotech, Little Chalfont, U.K.) was added to each well. The cells were harvested onto glass fiber filters, and the radioactivity incorporated in the cells was measured using a Wallac Betaplate liquid scintillation counter (Beckman, Fullerton, CA). The results were expressed as the mean cpm of triplicate cultures ± SEM.

Analysis of the gene expression by real-time quantitative PCR

Total RNA was extracted using Trizol (Molecular Research Center, Cincinnati, OH). Two micrograms of total RNA was reverse transcribed using the Superscript Reverse Transcription system (Takara, Shiga, Japan). The levels of mRNA expression were estimated using real-time quantitative PCR with LightCycler FastStart DNA master SYBR green I (Takara), according to the manufacturer’s instructions. The sequences of PCR primers for mouse and human are shown in Tables I and II, respectively.

Detection of phospho-STATs and intracellular cytokines by flow cytometry

Intracellular analysis of cytokine was performed using flow cytometry on a FACSCalibur (Becton Dickinson, San Jose, CA). The CD4+ T cells from mice were plated at 1 × 106 cells/well in a 24-well plate (Nunc, Roskilde, Denmark). The cells were incubated with 10 ng/ml of each cytokine for 4 h, and then incubated with anti–CD4–FITC, anti–CD8–PE, anti–CD25–PE-Cy7, and anti–Foxp3–APC-Cy7 Abs (all from eBioscience, San Diego, CA). The cells were fixed with 4% paraformaldehyde and permeabilized with Cytofix/Cytoperm Plus (BD Pharmingen). The cells were incubated with biotinylated anti–pSTAT1, anti–pSTAT3, anti–pSTAT6, and anti–pSTAT5 Abs (all from Cell Signaling Technology, Danvers, MA) overnight at 4°C. The cells were incubated with streptavidin–Alexa Fluor 647 (BD Pharmingen) and biotinylated anti–pSTAT1, anti–pSTAT3, anti–pSTAT6, and anti–pSTAT5 Abs (all from Cell Signaling Technology). The final color product was developed using 3,3-diaminobenzidine chromogen (DAKO, Carpinteria, CA). The sections were counterstained with hematoxylin. Images were captured using a DP71 digital camera (Olympus, Center Valley, PA) attached to an Olympus BX41 microscope at 400 magnification. For histologic evaluation of CIA, sections were evaluated in a blind manner, as has been described previously. The scores were evaluated as previously described (23).

Table I. Mouse primer sequences

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*GenBank is available online at http://www.ncbi.nlm.nih.gov/GenBank/*

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Denmark) and stimulated with 1 μg/ml plate-bound anti-CD3 mAb and 1 μg/ml anti-CD28 mAb (BD Pharmingen) for 3 d under Th17 cell-polarizing conditions (10 μg/ml anti–IFN-γ, 10 μg/ml anti–IL-4, 2 ng/ml TGF-β, 20 ng/ml IL-6). After 3 d of stimulation, the cells were restimulated with 25 ng/ml PMA and 250 ng/ml ionomycin (both from Sigma, St. Louis, MO) in the presence of GolgiStop (BD Pharmingen) for 5 h. The cells were stained with PerCp-conjugated anti-CD4 Ab (BD Pharmingen), then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) using a Foxp3 staining buffer kit according to the manufacturer’s instructions. Cells were stained with PE-conjugated anti–Foxp3 and FITC-conjugated anti–IL-17 (all from eBioscience, San Diego, CA).

To stain phospho-STATs, we pretreated splenocytes from the DBA/1J mice for 30 min with ATRA. The cells were cultured for 15, 30, and 60 min under conditions for Th17 cell differentiation, and they were stained with PE-conjugated anti–phospho-STAT3 (Y705) and anti–phospho-STAT5 (Y694; all from BD Pharmingen), using the Fixation Buffer and Perm Buffer III according to the manufacturer’s instructions. All the data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Immunoblotting**

Freshly isolated splenocytes or PMA (25 ng/ml) and ionomycin (250 ng/ml)-stimulated splenocytes from the mice treated with ATRA or vehicle were lysed with RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Lysates (50 μg) were electrophoresed and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 and incubated overnight at 4°C with the indicated primary antibodies. After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies and visualized with enhanced chemiluminescence reagents (PerkinElmer, Woburn, MA).

**Table II. Human primer sequences**

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<sup>a</sup>GenBank is available online at http://www.ncbi.nlm.nih.gov/genbank/.

**FIGURE 1.** Treatment with ATRA suppresses inflammatory arthritis in CIA mice. (A) A reduction in arthritis score and arthritis incidence in CIA mice via ATRA. The mice were immunized with CII. Seven days after immunization, the mice were treated with i.p. injections of ATRA (0.5 mg/kg) or vehicle three times per week for 9 wk. The results are shown as the mean arthritis score ± SD (left panel) and arthritis incidence (right panel). (B) Histologic examinations of the joints from the CIA mice treated with ATRA or vehicle. The mice were killed on day 49 after immunization. Tissue sections from the joints of each mouse were stained with H&E, toluidine blue, and safranin O. The joints from the mice treated with ATRA show mild erosive arthritis, whereas the joints from mice treated with vehicle show markedly erosive and destructive arthritis. The representative photographs from each group are shown. Original magnification ×400 or ×200, as indicated. The histologic scores of inflammation and cartilage damage in mice treated with ATRA (n = 10) or vehicle (n = 10) are shown in the right graph. Data are expressed as the mean ± SD. ***p < 0.01 compared with the vehicle-treated control group. (C) The tissue sections from the joints of the CIA mice treated with ATRA and vehicle were stained with the anti-nitrotyrosine, anti-iNOS, anti–TNF-α, anti–IL-1β, and anti–IL-6 or isotype control Abs. The cells stained with each Ab are shown in brown. Original magnification ×400.
were washed once with cold PBS, and the total proteins were extracted with lysis buffer (1% Nonidet P-40, PMSF, 2 mM sodium vanadate, 0.1% sodium deoxycholate, and protease inhibitor mixture; Roche Applied Science, Mannheim, Germany). The harvested lysates were centrifuged for 10 min at 4˚C to pellet cellular debris. The supernatants were removed and stored at −70˚C. The protein lysate (50 μg) was loaded on 10% SDS-PAGE, followed by transfer to nitrocellulose membranes (Invitrogen Life Technologies). The blots were then blocked with 5% nonfat dry milk in TBST for 1 h at room temperature; then the blots were incubated overnight at 4˚C with Abs specific for phospho-STAT3 (Y705, S727). After washing with TBST, the blots were incubated with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary Abs and the bands were revealed with ECL reagents (Amersham Biosciences, Piscataway, NJ). After stripping, the total anti-STAT3 was used as a loading control.

**In vitro osteoclastogenesis**

Bone marrow-derived monocyte/macrophage (BMM) cells were isolated from the tibia and femur of the mice. The cells were incubated with α-MEM (Invitrogen, Burlingame, CA) containing antibiotics and 10% heat-inactivated FBS for 12 h to separate the floating cells and adherent cells. The floating cells were seeded on 48-well plates at 1 × 10⁵ cells/well, and they were cultured in the presence of 10 ng/ml recombinant human (rh) M-CSF (R&D Systems, Minneapolis, MN) with MEM (alpha-MEM). Three days later, the washed out nonadherent cells and preosteoclasts were further cultured in the presence of 25 ng/ml M-CSF, 30 ng/ml RANKL, and various concentrations of ATRA for 4 d, to generate osteoclasts.

The PBMCs obtained from three healthy human volunteers were separated from theuffy coats using Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). The PBMCs with the RBCs removed were seeded onto 24-well plates at 5 × 10⁵ cells/well and incubated at 37˚C for 2 h to separate the floating and adherent cells. The adherent cells were washed with sterile PBS, and they were cultured with 100 ng/ml M-CSF. After 3 d, these cells were further cultured in the presence of 25 ng/ml M-CSF, 30 ng/ml RANKL, and various concentrations of ATRA for 9 d. On day 3, the medium was replaced with fresh medium containing M-CSF, RANKL, and ATRA. Informed consent was obtained from all the participating subjects. This study was reviewed and approved by our Institutional Review Board for human studies.

**Tartrate-resistant acid phosphatase staining**

Tartrate-resistant acid phosphatase (TRAP) staining was performed using a commercial kit (Sigma-Aldrich), according to the manufacturer’s instructions with omitting counterstaining with hematoxylin. The TRAP+ multinucleate cells with three or more nuclei were considered osteoclasts. The numbers of osteoclasts were determined according to the method described by Bendele et al. (24). All the histologic assessments were made by three independent blinded observers.

**Statistical analysis**

Statistical analyses were performed by using SAS software (Version 9). The experimental values are presented as means ± SD. Comparisons of numerical data between two groups were performed by Student t tests or Mann–Whitney U test. Differences in the mean values of various groups were analyzed by using ANOVA with a post hoc test. The p values <0.05 (two-tailed) were considered significant.

**Results**

**ATRA suppresses the inflammatory arthritis in CIA mice**

We investigated whether treatment with ATRA would suppress the rheumatoid inflammation and joint destruction in CIA mice. The results showed that i.p. injections of ATRA (0.5 mg/kg) reduced the arthritic score and arthritis incidence compared with i.p. injections of vehicle (Fig. 1A). We also delayed ATRA treatment to be able to determine the impact of ATRA on established disease. The CIA mice (n = 5) treated with ATRA, beginning on day 21 (3 wk) after the primary immunization, showed the equivalent arthritis score as the CIA mice (n = 5) treated with ATRA, beginning on day 7 (1 wk) (data not shown). On histologic examination of the joints, it was found that the paws and ankles of the ATRA-treated mice exhibited a lower degree of inflammation and cartilage damage compared with that of the vehicle-treated mice, as determined on day 49 after immunization (Fig. 1B). In addition, the ATRA-treated mice showed markedly decreased expressions of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, and oxidative stress markers such as nitrotyrosine and iNOS, which was demonstrated by immunohistochemistry (Fig. 1C).

**ATRA increased Foxp3-expressing regulatory T cell populations and decreased Th17 cells in CIA mice**

We next investigated whether ATRA would affect the population of new Th cell subsets, that is, regulatory T cells and Th17 cells. We evaluated the expression of IL-17, which is the main cytokine that characterizes Th17 cells (25), and IL-21, which is also produced by Th17 cells and plays important roles in the regulation of Th17 cells in arthritic joints. As shown in Fig. 2A, the CIA mice...
treated with ATRA showed profoundly decreased expressions of IL-17 and IL-21 compared with the mice treated with vehicle. We next examined the numbers of CD4+CD25+Foxp3+ regulatory T cells and decreased numbers of Th17 cells compared with that of the mice treated with vehicle (Fig. 2B). The mRNA levels of IL-17 and Foxp3, the latter of which is the master regulator in the development and function of regulatory T cells (26), in the splenocytes of CIA mice were measured in the presence of CII stimulation for 72 h. As expected, the mice treated with ATRA showed an increased expression of Foxp3 and a decreased expression of IL-17 (Fig. 2C). The results showed that the splenocytes obtained from the mice treated with ATRA showed decreased T cell proliferative responses to CII compared with those treated with vehicle (Fig. 2D).

**ATRA induces Foxp3 and represses IL-17 in the CD4+ T cells in mice**

We examined the effect of ATRA on Th17 cell differentiation in vitro. To investigate the role of ATRA under conditions favoring the development of Th17 cells, we cultured the splenocytes in the presence of anti-CD3, TGF-β, and IL-6 with or without ATRA. After stimulation under conditions favoring the development of Th17 cells, we found that the number of CD4+CD25+ cells expressing Foxp3 was substantially augmented after the addition of ATRA, whereas the number of IL-17–expressing CD4+ cells was decreased (Fig. 3A). There was no evidence of increased cell death in the ATRA-treated group (data not shown). We also checked the mRNA levels of Foxp3, IL-17, IL-21, and IL-10 in the splenocytes and lymph node cells obtained from the CIA mice treated with ATRA or vehicle. As shown in Fig. 3B, the ATRA-treated mice showed increased expression of Foxp3 and decreased expression of IL-17 in both the splenocytes and lymph node cells after in vitro stimulation with PMA and ionomycin. In addition, the ATRA-treated mice showed a decreased expression of IL-21 and an increased expression of IL-10 in the splenocytes. Intraacellular flow cytometry analysis also indicated that the ATRA-treated mice showed decreased expression of IL-17 in the splenocytes after in vitro stimulation with PMA and ionomycin.

The splenocytes isolated from the mice treated with ATRA or vehicle were treated with or without PMA and ionomycin, to investigate the signaling pathway that might mediate the ATRA-induced regulation of Th17 cells and regulatory T cells. The expressions of pSTAT3 were evaluated by Western blot analysis. The results showed that there was no difference in the expression of pSTAT3 between the mice treated with ATRA or vehicle (Fig. 3C). In addition, CD4+ T cells isolated from normal DBA/1J mice were cultured under Th17 cell-inducing cytokine conditions with or without ATRA. The expressions of pSTAT3 and pSTAT5 were determined by flow cytometry. The gray zone indicates isotype; gray line represents without ATRA; black line represents with ATRA.

**FIGURE 3.** ATRA induces Foxp3 and represses IL-17 in the CD4+ T cells in mice independent of the STAT3 or STAT5 pathway. (A) CD4+ T cells isolated from the spleen from DBA/1J mice were cultured under Th17 cell-inducing cytokine conditions in the presence or absence of ATRA (1 μM). Seventy-two hours later, the cells were stained with Abs against IL-17 and Foxp3 as described in Materials and Methods. (B) The pooled splenocytes and draining lymph node cells from the CIA mice treated with ATRA or vehicle were cultured with PMA (25 ng/ml) and ionomycin (250 ng/ml) for 4 h. The expressions of Foxp3, IL-17, IL-21, and IL-10 were determined by real-time PCR (upper panel). The pooled splenocytes from the mice treated with ATRA or vehicle were cultured with PMA (25 ng/ml) and ionomycin (250 ng/ml) for 4 h. The expression of IL-17 was determined by intracellular flow cytometry analysis (lower panel). *p < 0.05, compared with the vehicle-treated group. (C) The expression of pSTAT3 (Y705, S727) in the freshly isolated splenocytes from the CIA mice treated with ATRA or vehicle, or in splenocytes after 4-h stimulation with PMA (25 ng/ml) and ionomycin (250 ng/ml) was determined by Western blot analysis. (D) CD4+ T cells isolated from the spleen from DBA/1J mice were cultured under Th17 cell-inducing cytokine conditions (10 ng/ml anti-IFN-γ, 10 μg/ml anti–IL-4, 2 ng/ml TGF-β, 20 ng/ml IL-6) with or without ATRA (1 μM). The expressions of pSTAT3 and pSTAT5 were determined by flow cytometry. Gray zone indicates isotype; gray line represents without ATRA; black line represents with ATRA.
ATRA attenuates humoral immune responses in mice

We next examined whether ATRA would affect humoral immune responses in mice. We first investigated the effect of ATRA on Ab production in vitro. As shown in Fig. 4A, in vitro treatment with ATRA attenuated the production of total IgG and IgG2a in splenocytes that were stimulated by LPS. We next examined the effect of ATRA on the Ag-specific humoral immune responses in vivo; we measured the CII-specific total IgG and IgG2a in the sera of CIA mice at three different time points. As shown in Fig. 4B, the serum levels of total IgG and IgG2a were significantly lower in the mice treated with ATRA, as determined at 21, 42, and 70 d after immunization.

GCs are sites within peripheral lymphoid tissues where intense mature B lymphocytes rapidly proliferate, differentiate into plasma cells, mutate through somatic hypermutation, and class switch during Ab production (27). Therefore, we examined the impact of ATRA on GC formation, the critical step in humoral immune response, in vivo. The results showed that the spleen tissues of ATRA-treated mice exhibit markedly decreased formation of GC compared with that of vehicle-treated mice (Fig. 4C).

ATRA inhibits osteoclastogenesis in CIA mice

Osteoclasts are primarily involved in the bone destruction of RA. RANKL is the key osteoclastogenic molecule expressed by osteoclastogenesis-supporting cells like activated CD4+ T cells, osteoblasts, and synoviocytes (28, 29), and it binds to its single receptor RANK, and thereby mediates osteoclastogenesis that leads to bone destruction. To examine the effect of ATRA on osteoclast formation, we performed TRAP staining on the tissue sections from the joints from mice treated with ATRA or vehicle. As shown in Fig. 5A, the joint tissues of ATRA-treated mice showed a markedly reduced formation of osteoclasts compared with that of vehicle-treated mice. The expressions of RANKL, RANK, and other osteoclastogenesis-related molecules such as CTR and NFATc1 were markedly decreased in ATRA-treated mice.

We checked the mRNA levels of osteoclastogenic markers in bone marrow cells (BMCs), which include the precursors of osteoclasts, in the mice treated with ATRA or vehicle. The results showed that the expressions of TRAP, osteoclast-associated receptor (OSCAR), and cathepsin K were reduced in the BMCs of ATRA-treated mice (Fig. 5B). The expressions of integrin β3, CTR, and NFATc1 did not show any difference between the groups (data not shown).

Our next experiments were done to examine whether ATRA inhibits osteoclast formation in vitro. The BMM cells were prepared from CIA mice; then they were stimulated with M-CSF and/or RANKL to induce osteoclastogenesis. The addition of ATRA markedly inhibited osteoclastogenesis. These findings were evaluated by counting the numbers of TRAP+ cells per well (Fig. 5C). The mRNA levels of various osteoclastogenic markers such as CTR, integrin β3, TRAP, OSCAR, NFATc1, and cathepsin K were also remarkably decreased by the addition of ATRA (Fig. 5D). Collectively, these results imply that ATRA inhibits osteoclastogenesis in CIA mice both in vitro and in vivo.

ATRA inhibits osteoclastogenesis in humans

To determine the effect of ATRA on osteoclast formation in humans, we first examined whether ATRA would affect the expression of RANKL, the leading player of osteoclastogenesis, in the CD4+ T cells and synoviocytes. As shown in Fig. 6A, treatment with ATRA dose-dependently repressed the expression of RANKL in CD4+ T cells obtained from the human healthy control subjects, which were stimulated by anti-CD3 Ab. In addition, ATRA also repressed the RANKL expression of the fibroblast-like synoviocytes from patients with RA (RA-FLS) stimulated by IL-1β (Fig. 6B). Moreover, ATRA reduced the production of diverse matrix metalloproteinases (MMPs; MMP-1, MMP-3, and MMP-13) in the RA-FLS (Fig. 6C). Furthermore, in the presence of low-dose RANKL and M-CSF, the addition of ATRA prevented human
monocytes from differentiating into mature osteoclasts, which was determined by TRAP staining (Fig. 7A). It also reduced the expressions of osteoclastogenic markers such as TRAP, cathepsin K, and MMP-9 (Fig. 7B). These findings suggest that ATRA inhibits osteoclastogenesis in humans.

Discussion

In this study, we have demonstrated that treatment with ATRA reduced the clinical and histologic scores in CIA mice, which is the prototype of an animal model of RA. We proposed several evidences indicating that there are three possible mechanisms by
which ATRA have an antiarthritic effect: one is the regulation between regulatory T cells and Th17 cells, another is the inhibition of humoral immune responses, and the third is inhibition of osteoclast formation.

A couple of decades ago, Mosmann et al. (30) proposed that CD4\(^+\) T cells differentiate into two subsets with reciprocal functions and patterns of cytokine secretion, and these two subsets are called Th1 and Th2 cells. This classic paradigm was maintained until distinct Th cells subsets, Th17 cells (25, 31) and regulatory T cells (32), were identified. Regulatory T cells, in which the expression of transcription factor Foxp3 occurs, play major roles in the maintenance of immunologic self-tolerance and immune homeostasis (32, 33). In contrast, Th17 cells are a unique CD4\(^+\) T cell subset, and these cells are characterized by the production of IL-17. It is well-known that Th17 cells and IL-17 play a critical role in the pathogenesis of various autoimmune diseases, including RA (34, 35), psoriasis (36), multiple sclerosis (37), inflammatory bowel disease (38), and asthma (39).

Therefore, it can be speculated that both Th17 cells and IL-17 should be promising therapeutic targets in autoimmune diseases. In fact, therapeutic strategies targeting Th17 cells and IL-17 have been tried in human autoimmune diseases (40–44). Humanized anti–IL-17 mAbs are currently in clinical trial for patients with RA (42) and psoriasis (44). In addition, tofacitinib, a novel JAK inhibitor, which inhibits IL-17 production by human CD4\(^+\) T cells (45), is in clinical trial for RA patients (41, 43).

In this study, we found that ATRA inhibited Th17 cells and promoted Foxp3-expressing regulatory T cells in vivo during the development of inflammatory arthritis, as well as in vitro. These findings suggest that reciprocal regulation between regulatory T cells and Th17 cells by retinoic acid is one of the underlying mechanisms of retinoic acid’s antiarthritic effect.

When it comes to the impact of retinoic acid on humoral immune responses, a few reports have shown experimental evidence that retinoic acid might promote humoral immune responses (46, 47). Smith et al. reported that Ag-specific IgG1 responses were drastically impaired in vitamin A-deficient mice (46). Morikawa et al. demonstrated that the addition of exogenous retinoic acid to human B cell cultures enhanced the terminal differentiation of B cells into plasma cells (47). On the contrary, our study showed that treatment with ATRA reduced Ab production both in vitro and in vivo. In addition, ATRA reduced GC formation during the development of inflammatory arthritis. These findings suggest that retinoic acid attenuates inflammatory arthritis in mice by inhibiting the humoral immune response. Further investigations will be required to clarify the precise effect of retinoic acid on humoral immune responses and the underlying mechanisms.
Bone destruction is critically important for the prognosis of patients with RA because it is closely related to functional disability. Osteoclasts are specialized bone-resorbing cells, and they are the major cells that have been deeply implicated in bone destruction. There have been several discordant reports regarding the effect of retinoic acid on osteoclasts (48–53). Some showed that retinoic acid increased the number or activity of osteoclasts (48, 49, 51), whereas others reported that retinoic acid inhibits osteoclast formation (50, 52, 53). These inconsistencies between studies might stem from the different culture systems or species of experimental animals. There were two recent reports showing that ATRA inhibits osteoclastogenesis in vitro (52, 53). Conaway et al. (52) demonstrated that ATRA inhibits the in vitro differentiation of hematopoietic osteoclast progenitor cells into osteoclast using mouse BMM cells, bone marrow macrophages, and spleen cells. Hu et al. (53) showed that in vitro treatment with ATRA inhibits the RANKL-stimulated osteoclast differentiation of human monocytes by suppressing RANK expression. In this study, we demonstrated that in vitro treatment with ATRA inhibits osteoclastogenesis both in mice and humans, similar to what was found in a couple of previous reports (52, 53). In addition, we also verified that ATRA inhibits osteoclastogenesis in vivo during the development of inflammatory arthritis in mice. Therefore, despite a few reports that showed that retinoic acid increased the number or activity of osteoclasts (48, 49, 51), it is likely that retinoic acid inhibits osteoclastogenesis.

Development of biologic agents, such as the TNF-α inhibitors, has brought about a profound paradigm shift in the treatment of RA. This is mainly because combinations of methotrexate, which is the prototype of conventional DMARDs, and TNF-α inhibitors such as infliximab, etanercept, and adalimumab have proved to be significantly superior to methotrexate alone for inhibiting radiologic progression, as well as for improving the signs and symptoms of disease (35–37). Thus, retinoic acid, which we proved to have antosteoclastogenic activity in this study, could be a good therapeutic strategy for the treatment with RA and especially in light of preventing bone destruction.

In conclusion, we have observed that ATRA improved the clinical course of arthritis in CIA mice. It induces the expansion of regulatory T cells and suppresses Th17 cells. ATRA also inhibits humoral immune responses and osteoclast formation in vivo during the development of inflammatory arthritis, as well as in vitro. These data suggest that ATRA might be one of the therapeutic candidate molecules for treating the patients with RA.

Disclosures
The authors have no financial conflicts of interest.

References


