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Necessity of Lysophosphatidic Acid Receptor 1 for Development of Arthritis

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Objective. **Lysophosphatidic acid (LPA) is a bioactive lipid that binds to a group of cell surface G protein–coupled receptors (LPA receptors 1–6 [LPA_{1-6}]) and has been implicated as an important mediator of angiogenesis, inflammation, and cancer growth. This study was undertaken to analyze the effects of LPA_1 on the development of arthritis.**

Methods. **Expression of LPA receptors on synovial tissue was analyzed by immunohistochemistry and quantitative reverse transcription–polymerase chain reaction. The effects of abrogation of LPA_1 on collagen-induced arthritis (CIA) were evaluated using LPA_1-deficient mice or LPA_1 antagonist. Migrating fluorescence-labeled CD11b+ splenocytes, which were transferred into the synovium of mice with CIA, were counted. CD4+ naive T cells were incubated under Th1-, Th2-, or Th17-polarizing conditions, and T helper cell differentiation was assessed. Osteoclast formation from bone marrow cells was examined.**

Results. **LPA_1 was highly expressed in the synovium of patients with rheumatoid arthritis (RA) compared with that of patients with osteoarthritis. LPA_1-deficient mice did not develop arthritis following immunization with type II collagen (CII). LPA_1 antagonist also ameliorated murine CIA. Abrogation of LPA_1 was associated with reductions in cell infiltration, bone destruction in the joints, and interleukin-17 production from CII-stimulated splenocytes. Infiltration of transferred CD11b+ macrophages from LPA_1-deficient mice into the synovium was suppressed compared with infiltration of macrophages from wild-type mice. LPA_1 antagonist inhibited the infiltration of macrophages from wild-type mice. Differentiation into Th17, but not Th1 or Th2, and osteoclast formation were also suppressed under conditions of LPA_1 deficiency or LPA_1 inhibition in vitro.**

Conclusion. **Collectively, these results indicate that LPA/LPA_1 signaling contributes to the development of arthritis via cellular infiltration, Th17 differentiation, and osteoclastogenesis. Thus, LPA_1 may be a promising target molecule for RA therapy.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammatory cell infiltration and bone destruction at multiple joints. The inflammation process in RA leads to synovial hyperplasia with proliferation of fibroblast-like synoviocytes (FLS), angiogenesis, and infiltration of inflammatory cells, including lymphocytes and macrophages (1,2). Biologic drugs, such as anti–tumor necrosis factor (anti-

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TNF) monoclonal antibody (mAb), have been reported to markedly improve arthritis and inhibit bone destruction (3,4). However, in some patients RA does not respond to the treatment, and biologic agents increase the risk of severe infection (5,6). Therefore, there remains a need for new RA therapies that are effective and safe compared with conventional treatments.

Lysophosphatidic acid (LPA) is a bioactive lipid that binds to a group of cell surface G protein–coupled receptors (LPA receptors 1–6 [LPA_{1-6}]) and has been implicated as an important mediator of angiogenesis, inflammation, and cancer growth (7–9). LPA is generated via hydrolysis of lysophosphatidylcholine by a secretory protein, autotaxin (ATX) (10), which exerts lysophospholipase D activity (11–13). In a bleomycin-induced lung injury/fibrosis model, mice lacking LPA_1 have been shown to have decreased fibroblast recruitment and vascular leak, and LPA_1 has been noted to be a therapeutic target for interstitial pneumonia (14). Messenger RNA (mRNA) for ATX and LPA_{1-3} is expressed on FLS from patients with RA, and LPA has been shown to induce cell motility and production of interleukin-6 (IL-6) and IL-8 in RA FLS (15–17). It was recently demonstrated that conditional genetic ablation of ATX in mesenchymal cells attenuates the development of arthritis in an animal model of RA (18).

These reports suggested that the ATX/LPA axis might contribute to the pathogenesis of RA. However, blocking of ATX should diminish LPA levels, which would then cause the reduction of signals from all LPA receptors (LPA_{1-6}) rather than selectively blocking one and allowing the activity of the others to remain intact. In addition, it was shown that ATX exerts blood vessel–stabilizing actions, and in fact, ATX-deficient mice die in utero due to severe vascular defects (19). Identification of the specific LPA receptor(s) involved in the pathogenesis of arthritis and elucidation of the mechanisms of LPA that contribute to arthritis could lead to the development of a new therapeutic target in RA. In this study we demonstrated important roles of LPA_1 in inflammatory cell infiltration, Th17 differentiation, and osteoclastogenesis and showed that abrogation of LPA_1 ameliorated arthritis, suggesting that LPA_1 should be investigated further as a potential target in the treatment of RA.

MATERIALS AND METHODS

Patient specimens. Synovial tissue (ST) and synovial fluid (SF) were obtained from 15 patients (1 male and 14 female) with RA that fulfilled the American College of Rheumatology/European League Against Rheumatism criteria (20) and from 10 patients (2 male and 8 female) with osteoarthritis (OA) who underwent total knee joint replacement. Among the RA patients, the mean ± SEM age was 66.6 ± 3.0 years, disease duration was 15.3 ± 3.0 years, and C-reactive protein level was 0.60 ± 0.23 mg/dl. Ten (67%) were positive for rheumatoid factor and 9 (60%) were positive for anti–citrullinated protein antibodies. The experimental protocol was approved by the ethics committee of the Tokyo Medical and Dental University, and all subjects provided informed consent according to Declaration of Helsinki principles.

Enzyme-linked immunosorbent assays (ELISAs) for ATX and anti–type II collagen (anti-CII) antibodies. The concentration of ATX in SF was determined by ELISA as described previously (21,22). Levels of IgG1, IgG2a, and IgG2b anti-CII in serum were measured by ELISA as described previously (23).

Immunohistochemistry. Paraffin-embedded ST (4-μm–thick sections) from RA and OA patients and from mice with collagen-induced arthritis (CIA) (see below) were deparaffinized and then immersed in 1 m*M* EDTA for 20 minutes at 99–100°C, removed from heat, and kept at room temperature for 20 minutes, followed by rinsing with Tris buffered saline–Tween. Endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 for 30 minutes. Sections were then blocked with 1% skim milk for 45 minutes and stained with rabbit anti-ATX polyclonal antibody (2 μg/ml; Cayman Chemical), anti-LPA_1 polyclonal antibody (10 μg/ml; LifeSpan Biosciences), or normal rabbit IgG (Sigma-Aldrich) as an isotype control, for 45 minutes at room temperature. Antibody binding was detected using an Envision kit (DakoCytomation).

For double immunofluorescence staining with CD68 or von Willebrand factor (vWF) and ATX or LPA_1, nonspecific binding was blocked with 1% skim milk and then the sections were incubated for 2 hours at room temperature with mouse anti-CD68 mAb (KP1) (9.4 μg/ml; DakoCytomation) or anti-vWF mAb (F8/86) (8 μg/ml; DakoCytomation). Subsequently, the samples were incubated with Alexa Fluor 488–conjugated goat anti-mouse IgG2a (Invitrogen) for 1 hour at room temperature. Next, the sections were incubated with rabbit anti-ATX, anti-LPA_1 polyclonal antibody, or isotype control as described above for 45 minutes at room temperature, and then with Alexa Flour 568–conjugated goat anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. The slides were examined using a fluorescence microscope (Biozero; Keyence).

Real-time reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was prepared from the ST of RA and OA patients and mice with CIA, and first-strand complementary DNA (cDNA) was synthesized. Quantitative real-time PCR was performed as described previously (24). The cDNA was amplified with primers for LPA_1 (sense 5′-ACC-CAA-TAC-TCG-GAG-ACT-GAC-TGT-3′, antisense 5′-CG-T-CAG-GCT-GGT-GTC-AAT-GA-3′), LPA_2 (sense 5′-TCA-TCA-TGG-GCC-AGT-GCT-ACT-3′, antisense 5′-GTG-GGA-GCT-GAG-CTC-TTT-GC-3′), LPA_3 (sense 5′-CTT-GAC-TGC-TTC-CCT-CAC-CAA-3′, antisense 5′-CGC-ATC-CTC-ATG-ATT-GAC-ATG-3′), LPA_4 (sense 5′-TCC-TCA-GTG-GCG-GTA-TTT-CAG-3′, antisense 5′-AAG-CAG-GTG-GTG-GTT-GCA-TT-3′), LPA_5 (sense 5′-GGT-GGT-

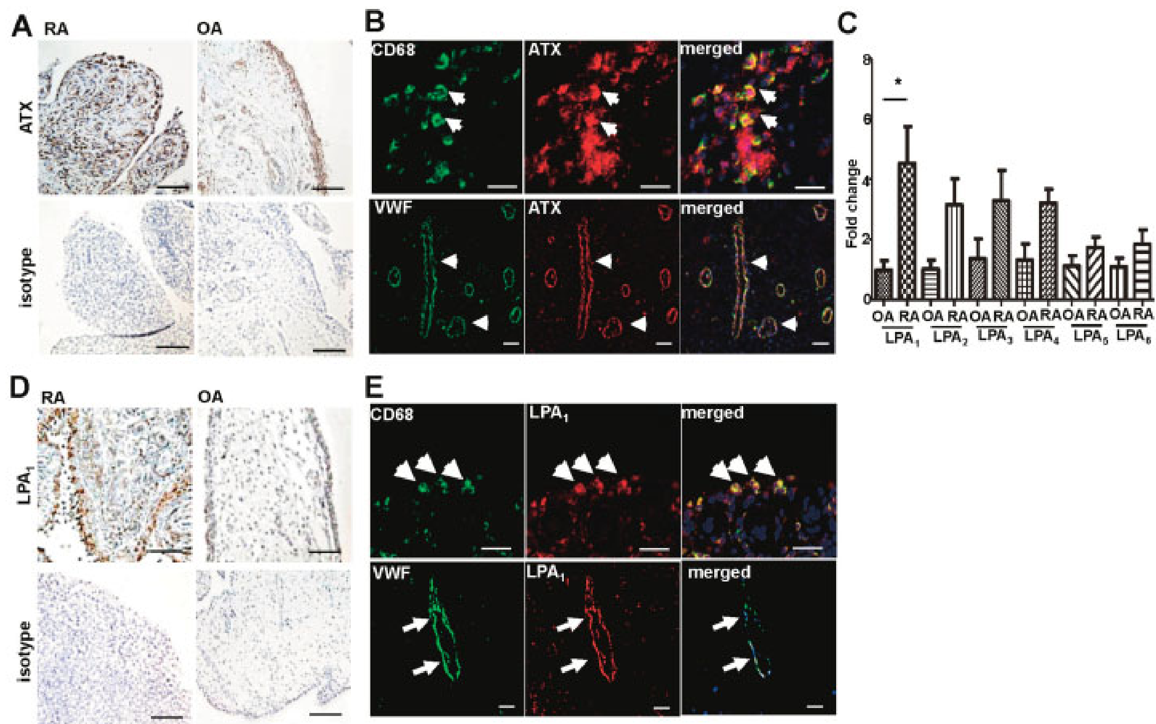

Figure 1. Increased expression of autotaxin (ATX) and lysophosphatidic acid receptor 1 (LPA$_1$) in rheumatoid arthritis (RA) synovium. **A,** Expression of ATX in the synovium of RA patients (n = 5) and osteoarthritis (OA) patients (n = 5) was analyzed by immunohistochemistry. **B,** RA synovium was double-stained with CD68 or von Willebrand factor (vWF) and ATX. **Arrows** and **arrowheads** indicate double-positive cells. **C,** Expression levels of LPA$_{1-6}$ mRNA in RA synovium (n = 5) and OA synovium (n = 5) were determined by real-time reverse transcription–polymerase chain reaction. Values are the mean ± SEM. $* = P < 0.05$. **D,** Expression of LPA$_1$ in the synovium of RA patients (n = 5) and OA patients (n= 5) was analyzed by immunohistochemistry. **E,** RA synovium was double-stained with CD68 or vWF and LPA$_1$. **Arrows** and **arrowheads** indicate double-positive cells. Bars in **A** and **D** = 100 μm; bars in **B** and **E** = 50 μm. Representative images are shown.

GAG-CGT-GTA-CAT-GTG-T-3′, antisense 5′-AGT-GGT-GCA-GTG-CGT-AG-TAG-GA-3′), LPA$_6$ (sense 5′-AGA-A-CC-AAA-AGA-AAT-GCA-AAG-ATT-G-3′, antisense 5′-ACG-GCG-GGT-GCA-CTT-C-3′), IL-17 (sense 5′-TCT-TTA-ACT-CCC-TTG-GCG-CA-3′, antisense 5′-GGT-AGT-CTG-AGG-GCC-TTC-TGG-3′), and 18S ribosomal RNA (rRNA) (sense 5′-AAC-CAG-ACA-AAT-CGC-TCC-AC-3′, antisense 5′-ACT-CAA-CAC-GGG-AAA-CCT-CA-3′) (used as an internal control to standardize the amount of sample mRNA), and the relative expression of real-time PCR products was determined.

Induction and assessment of CIA. CIA was induced in LPA$_1$-deficient (25) or wild-type (WT) mice on a C57BL/6 background, as described previously (26). Mice were immunized with chicken CII on day 0 followed by a booster immunization on day 21, and were killed on day 38. CIA was also induced in DBA1/J mice, as described previously (23). Mice were immunized with bovine CII on day 0 followed by a booster immunization on day 21, and were killed on day 36.

Clinical arthritis in each paw was scored on a scale of 0–4 (0 = normal, 1 = erythema and swelling of 1 digit, 2 = erythema and swelling of 2 digits or erythema and swelling of the ankle joint, 3 = erythema and swelling of >3 digits or swelling of 2 digits and the ankle joint, and 4 = erythema and severe swelling of the ankle, foot, and digits with deformity). The scores of the 4 paws were summed to determine a total arthritis score for each animal (maximum possible score 16). The hind paw of each mouse was dissected and examined histologically after hematoxylin and eosin staining. Histologic scores of 0–2 were assigned (0 = no inflammation, 1 = focal inflammatory infiltration, and 2 = severe and diffuse inflammatory infiltration). Radiographic findings in the bilateral second through fourth metatarsophalangeal joints were scored 0–2 according to a previously reported classification system for bone erosion (0 = not obvious, 1 = erosion <0.3 mm in diameter, and 2 = erosion >0.3 mm in diameter) (23). Six-μm–thick frozen hind paw sections were stained with tartrate-resistant acid phosphatase (TRAP; Kureha Special Laboratory), and TRAP-positive osteoclasts in the ankle joint were counted. The experimental protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Treatment of CIA with an LPA$_1$ antagonist. DBA1/J mice with CIA were administered an LPA$_1$ antagonist (LA-01; Ono Pharmacological) (23,27) or vehicle at 200 mg/kg/day or 60 mg/kg/day by oral gavage, twice daily from day 21 to day 35. To determine its therapeutic effects, LA-01 (200 mg/kg/day) was administered from day 27 to day 35, and clinical signs of

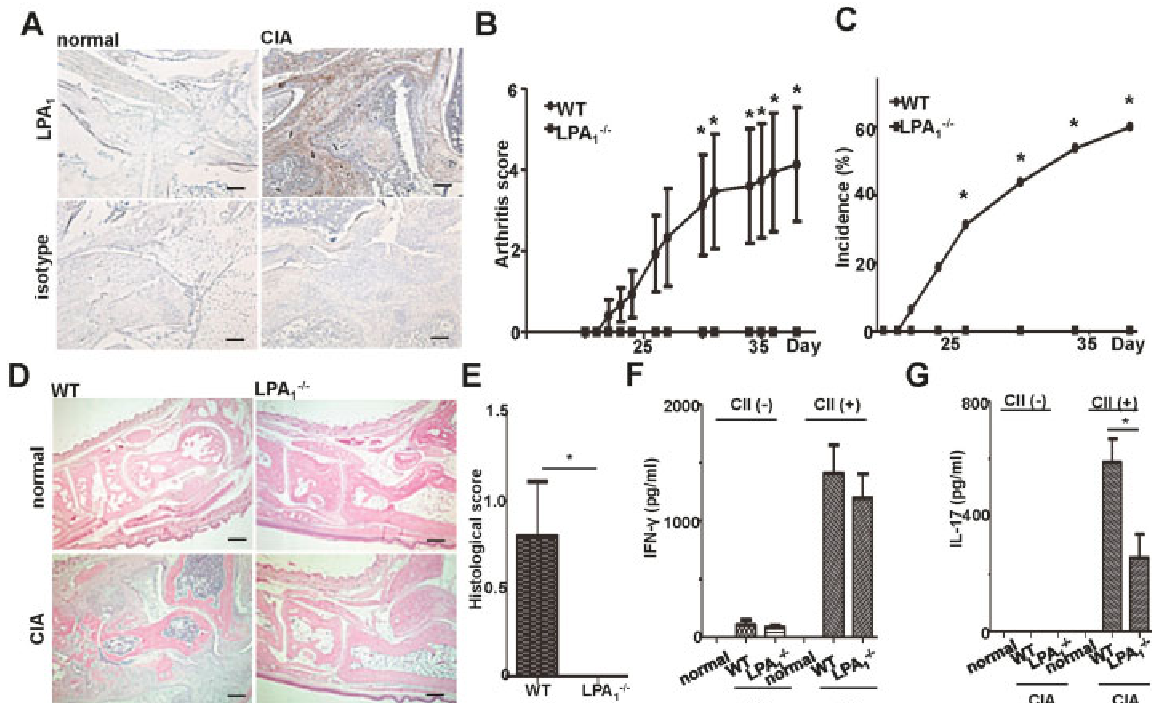

Figure 2. Inhibition of arthritis development in lysophosphatidic acid receptor 1 (LPA_1)–deficient mice. Collagen-induced arthritis (CIA) was induced in LPA_1-deficient mice on a C57BL/6 background (n = 17) and in wild-type (WT) mice (n = 16) by immunization with chicken type II collagen (CII) on days 0 and 21. **A,** Expression of LPA_1 in the synovium of CII-immunized and normal mice, analyzed by immunohistochemistry. **B** and **C,** Clinical arthritis score (**B**) and arthritis incidence (**C**) in LPA_1-deficient and WT mice. **D,** Histologic features in hematoxylin and eosin–stained ankle joints of normal and CII-immunized LPA_1-deficient and WT mice. **E,** Histologic scores in LPA_1-deficient and WT mice. **F** and **G,** Concentrations of interferon-γ (IFNγ) (**F**) and interleukin-17 (IL-17) (**G**) in the culture supernatants of CII-stimulated splenocytes from LPA_1-deficient and WT mice, determined by enzyme-linked immunosorbent assay. Values in **B, E, F,** and **G** are the mean ± SEM. $* = P < 0.05$. Bars in **A** and **D** = 300 μm. Representative images are shown.

arthritis were assessed. LPA_1, LPA_2, and LPA_3 share 45–48% amino acid homology in mice and comprise the endothelial cell differentiation gene (Edg) family LPA receptors (28). LA-01 is a specific LPA_1 receptor antagonist (50% inhibition concentration 0.086 μmoles/liter, 2.8 μmoles/liter, and 0.90 μmoles/liter for LPA_1, LPA_2, and LPA_3, respectively). LPAs 4–6 are classified as non–Edg family LPA receptors and are structurally distant from the Edg family LPA receptors (28). LPA_4, LPA_5, and LPA_6, respectively, share only 22%, 23%, and 22% amino acid homology with LPA_1.

Measurement of cytokine production by CII-stimulated splenocytes. Splenocytes from mice with CIA were harvested and the cells (1×10^6) were cultured in 48-well plates in RPMI medium with 10% fetal bovine serum (Sigma-Aldrich) supplemented with 50 μg/ml denatured (100°C, 10 minutes) CII (23). Seventy-two hours later, concentrations of interferon-γ (IFNγ) and IL-17 in the culture supernatant were measured by ELISA according to the instructions of the manufacturer (R&D Systems).

Migration of CD11b+ splenocytes into the synovium. CD11b+ splenocytes from mice with CIA were purified using MACS MicroBeads (purity >95%; Miltenyi Biotec) and labeled with CellTracker Orange according to the protocol supplied by the manufacturer (Molecular Probes). The labeled cells (1×10^7) were transferred into the tail vein of mice with CIA on day 25. Twelve hours before and 30 minutes before the transfer and 12 hours after the transfer, recipient mice were treated with LA-01 (100 mg/kg) or saline. Twenty-four hours after the transfer, labeled cells in the synovium were counted under fluorescence microscopy (29).

T helper cell differentiation in vitro. Naive CD4+ T cells were purified from the spleen using a mouse T cell negative isolation kit (Invitrogen), after which CD8+ and CD44+ cells were further depleted by magnetic-activated cell sorting (purity of CD4+CD44− cells >95%). The purified cells were stimulated with plate-bound anti-CD3 mAb (2 μg/ml) and anti-CD28 mAb (2 μg/ml) (both from eBioscience), with or without LA-01 (10 n*M*), for 3 days under various polarizing conditions. The number of viable cells among T cells treated with LA-01 for 3 days was >95% of the number of viable cells among those treated with vehicle, suggesting that culture with LA-01 had no significant effect on T cell viability.

The conditions for analysis of different T helper cell subsets were as follows: for Th0 (neutral conditions), anti–IL-4 mAb (11B11) (10 μg/ml) (BioLegend) and anti-IFNγ mAb (XMG1.2) (10 μg/ml) (eBioscience); for Th1, anti–IL-4 mAb

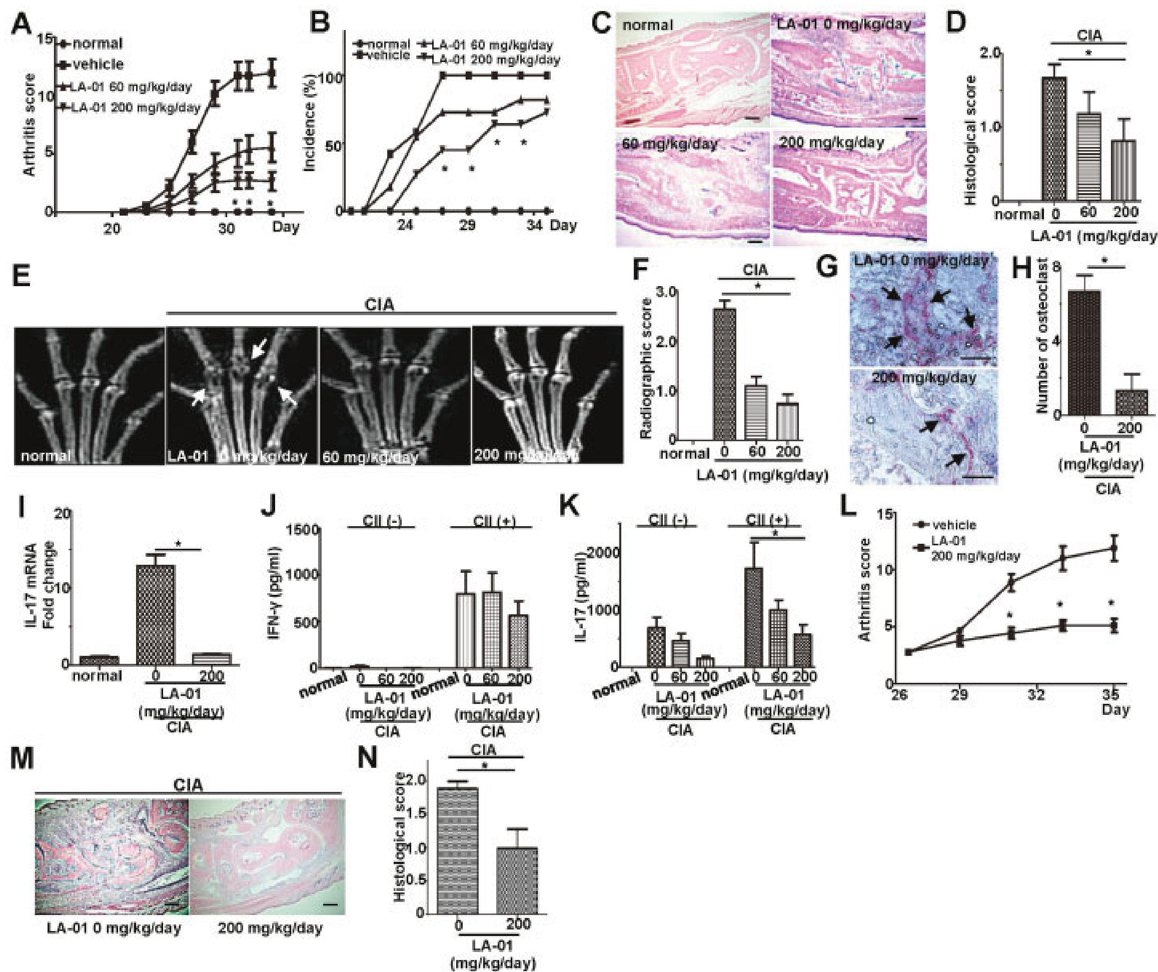

Figure 3. Amelioration of CIA by the LPA$_1$ antagonist LA-01. DBA1/J mice were immunized with bovine CII on days 0 and 21 and LA-01 was administered twice daily from day 21 to day 35 (**A–K**) or, in experiments to determine its therapeutic effects, was administered from day 27 to day 35 to mice with established CIA (**L–N**). **A** and **B,** Clinical arthritis score (**A**) and arthritis incidence (**B**) in CII-immunized mice treated with LA-01 60 mg/kg/day (n = 12), LA-01 200 mg/kg/day (n = 12), or vehicle (n = 12) and in control mice (n = 3). * = $P < 0.05$, LA-01 200 mg treatment versus vehicle treatment. **C** and **D,** Hematoxylin and eosin (H&E)–stained ankle joints (**C**) and histologic scores (**D**) of CII-immunized mice treated with LA-01 60 mg/kg/day, LA-01 200 mg/kg/day, or vehicle and of control mice. **E** and **F,** Radiographs of the metatarsophalangeal joints (**E**) and radiographic scores (**F**). **Arrows** in **E** indicate bone erosion. **G** and **H,** Tartrate-resistant acid phosphatase (TRAP)–stained ankle joints (**G**) and numbers of TRAP-positive osteoclasts in the ankle joints (**H**). **Arrows** in **G** indicate TRAP-positive osteoclasts. **I,** Expression levels of IL-17 mRNA in the synovium of CII-immunized mice treated with LA-01 (200 mg/kg/day) (n = 5) or vehicle (n = 5) and of control mice (n = 3), determined by real-time reverse transcription–polymerase chain reaction. **J** and **K,** Concentrations of IFNγ (**J**) and IL-17 (**K**) in CII-stimulated splenocytes from mice with CIA treated with LA-01 60 mg/kg/day, LA-01 200 mg/kg/day, or vehicle and from control mice, determined by enzyme-linked immunosorbent assay. **L,** Clinical arthritis score in mice with CIA administered LA-01 from day 27 to day 35. **M** and **N,** H&E-stained ankle joints (**M**) and histologic scores (**N**) on day 35. Values in **A, D, F, H–L,** and **N** are the mean ± SEM. * = $P = 0.05$. Bars in **C** and **M** = 300 μm; bars in **G** = 600 μm. Representative images are shown. See Figure 2 for other definitions.

(10 μg/ml) and IL-12 (10 ng/ml) (PeproTech); for Th2, anti-IFNγ mAb (10 μg/ml) and IL-4 (10 ng/ml); for Th17, anti-IFNγ mAb (10 μg/ml), anti–IL-4 mAb (10 μg/ml), and IL-6 (30 ng/ml), transforming growth factor β (2.5 ng/ml), IL-21 (80 ng/ml), IL-1α (10 ng/ml), and TNFα (10 ng/ml) (all from PeproTech) (30). Activated cells were restimulated with phorbol-12-myristate-13-acetate (100 ng/ml; Sigma-Aldrich) and ionomycin (0.5 μg/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences) for 4 hours before intracellular staining. For intracellular cytokine staining, allophycocyanin-conjugated anti-mouse IFNγ mAb, phycoerythrin-conjugated anti-mouse IL-4 mAb (11B11), and phycoerythrin-conjugated anti-mouse IL-17 mAb (TC11-18H10; BD Biosciences) were used. Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences).

Expression of mRNA for T-bet, GATA-3, and retinoic

acid receptor–related orphan nuclear receptor γt (RORγt) was quantified by real-time RT-PCR using primers for T-bet (sense 5′-TCC-TGC-AGT-CTC-TCC-ACA-AGT-3′, antisense 5′-CAG-CTG-AGT-GAT-CTC-TGC-GT-3′), GATA-3 (sense 5′-CCT-ACC-GGG-TTC-GGA-TGT-AAG-T-3′, antisense 5′-AGT-TCG-CGC-AGG-ATG-TCC-3′), and RORγt (sense 5′-AGG-GGA-TTC-AAC-ATC-AGT-GC-3′, antisense 5′-TGC-AAG-ACT-CAT-CGA-CAA-GG-3′); 18S rRNA was used as an internal control.

Osteoclast differentiation. Murine bone marrow cells derived from C57BL/6 mice (5×10^4 cells per well of a 96-well plate) cultured with macrophage colony-stimulating factor (M-CSF) (10 ng/ml; R&D Systems) for 2 days were used as monocyte/macrophage precursor cells (bone marrow–derived macrophages [BMMs]), which were further cultured for 3 days with RANKL (70 ng/ml; PeproTech) and M-CSF (10 ng/ml) with or without LA-01 (10 n*M*) (31). The number of viable cells among cells treated with LA-01 for 3 days was >95% of the number of viable cells among those treated with vehicle. Cells were stained with a TRAP staining kit (Hokudo). The total number of TRAP-positive multinucleated cells (≥3 nuclei) per well was counted under light microscopy. Murine BMMs were also seeded onto plates coated with calcium phosphate (Biocoat Osteologic; BD Biosciences), and the area of resorption lacunae was determined under light microscopy and analyzed using ImageJ software (National Institutes of Health).

Statistical analysis. Data are expressed as the mean ± SEM. The significance of differences between groups was assessed by Student's *t*-test or chi-square test. *P* values less than 0.05 were considered significant.

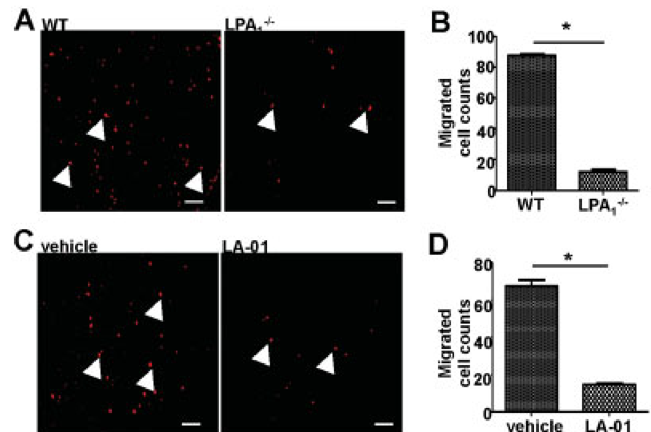

Figure 4. Inhibition of CD11b+ splenocyte migration into the synovium by abrogation of LPA_1. **A** and **B,** Fluorescence-labeled CD11b+ splenocytes from LPA_1-deficient and WT mice were transferred into WT mice with CIA on day 25, and 24 hours after transfer the number of labeled cells that migrated into the synovium was evaluated histologically (**A**) and counted under fluorescence microscopy (**B**). **Arrowheads** in **A** indicate migrated CD11b+ cells. **C** and **D,** Fluorescence-labeled CD11b+ splenocytes from WT mice were transferred into WT mice with CIA on day 25. Twelve hours before, 30 minutes before, and 12 hours after transfer the recipient mice were treated with the LPA_1 antagonist LA-01 (100 mg/kg) or saline as a control, and 24 hours after transfer the number of labeled cells that migrated into the synovium was evaluated histologically (**C**) and counted under fluorescence microscopy (**D**). **Arrowheads** in **C** indicate migrated CD11b+ cells. Values in **B** and **D** are the mean ± SEM. * = $P < 0.05$. Bars in **A** and **C** = 50 μm. Representative images are shown. See Figure 2 for definitions.

RESULTS

Expression of ATX and LPA receptors in the RA synovium. Expression of ATX was analyzed by immunohistochemistry and ELISA. ATX was highly expressed in RA synovium compared with OA synovium (Figure 1A), as well as in RA SF (mean ± SEM 0.60 ± 0.093 mg/liter; n = 15) compared with OA SF (0.27 ± 0.045 mg/liter; n = 10) ($P < 0.05$), similar to previously reported findings (18). In RA ST, ATX was expressed on stromal cells, including CD68+ macrophages and vWF-positive endothelial cells (Figure 1B).

Next, we analyzed the expression of LPA receptors in the RA synovium. As demonstrated by quantitative RT-PCR, expression of LPA_1 mRNA was significantly higher in RA ST than in OA ST (Figure 1C). Immunohistochemistry analysis revealed LPA_1 expression on synovial lining and sublining cells, and this expression was also higher compared with OA synovium (Figure 1D). Double staining showed that LPA_1 was expressed on stromal cells, including CD68+ macrophages and vWF-positive endothelial cells in the RA synovium (Figure 1E).

Necessity of LPA_1 for development of CIA in mice. Expression of LPA_1 was markedly increased in the synovium of mice with CIA compared with normal synovium (Figure 2A). To examine the role of LPA_1 in arthritis, we evaluated CIA in LPA_1-deficient mice. Interestingly, mice lacking LPA_1 did not develop arthritis (incidence 0% on day 38, versus 60% in WT mice) (Figures 2B and C). Histologically, no inflammatory cell infiltration was noted in the ankle joints of LPA_1-deficient mice, whereas abundant infiltration was observed in WT mice (Figures 2D and E).

Since T helper cell differentiation influences the development of CIA (32,33), we measured the production of IFNγ and IL-17 by CII-stimulated splenocytes from mice with CIA. Production of IFNγ and IL-17 was increased in splenocytes from WT mice upon CII stimulation. CII-induced production of IL-17 was decreased in splenocytes from LPA_1-deficient mice compared to that in splenocytes from WT mice, whereas the difference in CII-induced IFNγ was not significantly different (Figures 2F and G). Serum anti-CII antibodies were also measured by ELISA and were detected in CII-immunized mice but not in normal mice. Levels of IgG1, IgG2a, and IgG2b anti-CII antibodies were not signifi-

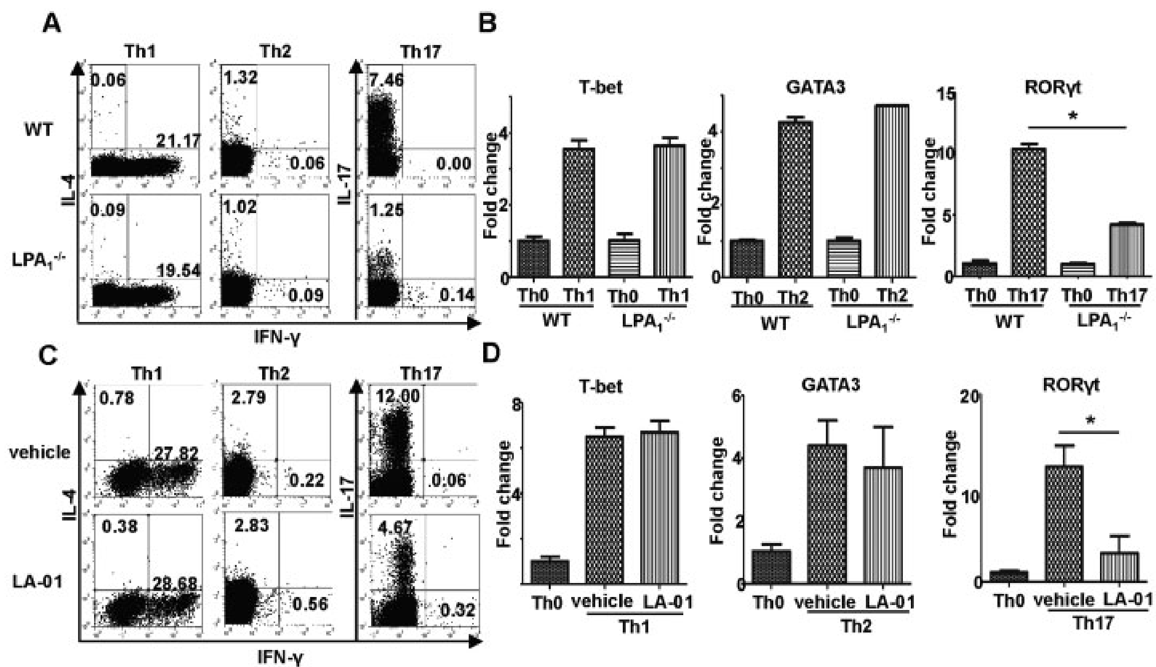

Figure 5. Deficiency of LPA_1 inhibits Th17 differentiation in vitro. **A** and **B,** CD4+ T cells from naive LPA_1-deficient and WT mice were cultured with anti-CD3 and anti-CD28 monoclonal antibodies (mAb) under Th1-, Th2-, or Th17-polarizing conditions. Proportions of IFNγ,- IL-4–, and IL-17–producing cells were analyzed by flow cytometry (**A**), and expression of mRNA for T-bet, GATA-3, and retinoic acid receptor–related orphan nuclear receptor γt (RORγt) was quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR) (**B**). **C** and **D,** CD4+ T cells from naive WT mice were cultured with anti-CD3 and anti-CD28 mAb and the LPA_1 antagonist LA-01 (10 n*M*) under Th1-, Th2-, or Th17-polarizing conditions. Proportions of IFNγ-, IL-4–, and IL-17–producing cells were analyzed by flow cytometry (**C**), and expression of mRNA for T-bet, GATA-3, and retinoic acid receptor–related orphan RORγt was quantified by RT-PCR (**D**).Values in **B** and **D** are the mean ± SEM. $* = P < 0.05$. See Figure 2 for other definitions.

cantly different between LPA_1-deficient and WT mice (data not shown).

We next analyzed the effect of LPA_1 antagonist (LA-01) in mice with CIA. LA-01 was administered orally twice daily from day 21 (the day of the second immunization) to day 35. Treatment with LA-01 (200 mg/kg/day) significantly reduced the arthritis score and incidence of arthritis (Figures 3A and B). On histologic examination, LA-01–treated animals also exhibited decreased cellular infiltration into the ankle joints on day 36 (Figures 3C and D). Radiologically, bone destruction was noted in the metatarsophalangeal joints of mice with CIA, and such destruction was diminished by LA-01 treatment (Figures 3E and F). Moreover, treatment with LA-01 reduced the number of TRAP-positive osteoclasts in the ankle joints of mice with CIA (Figures 3G and H). In addition, LA-01 suppressed the level of IL-17 mRNA in the synovium (Figure 3I) as well as the production of IL-17, but not IFNγ, by CII-stimulated splenocytes from mice with CIA (Figures 3J and K), similar to findings in LPA_1-deficient mice. Levels of serum IgG1, IgG2a, and IgG2b anti-CII antibodies were unaffected by administration of LA-01 (data not shown). To examine the effect of LPA_1 antagonist on established arthritis, LA-01 was administered from day 27, after the development of arthritis, to day 35. Treatment with LA-01 (200 mg/kg/day) significantly inhibited clinical arthritis (Figure 3L) and also suppressed inflammatory cell infiltration into the inflamed joints (Figures 3M and N).

These results indicated that LPA_1 contributes to inflammatory cell infiltration, bone erosion, and IL-17 production in vivo. Collectively, they suggest that the LPA/LPA_1 cascade might play an important role in the development of arthritis.

Inhibition of inflammatory cell infiltration by abrogation of LPA_1. To investigate the role of LPA_1 in inflammatory cell infiltration into the synovium, fluorescence-labeled CD11b+ splenocytes from LPA_1-deficient or WT mice were transferred into WT mice with CIA, and 24 hours later the number of cells that migrated into the synovium was counted. We have reported previously that most migrating CD11b+ splenocytes are F4/80-positive macrophages (29). Migration of CD11b+ splenocytes derived from LPA_1-

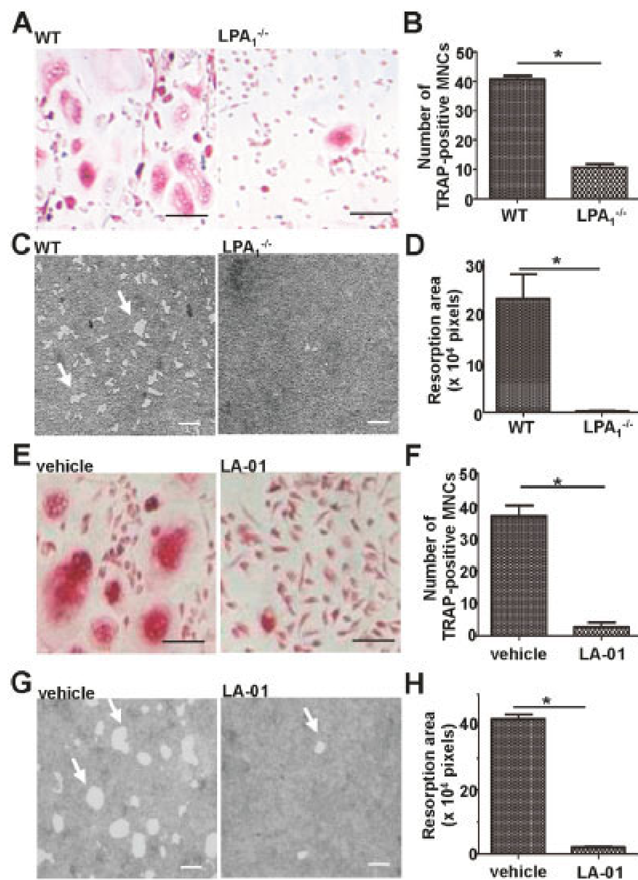

Figure 6. LPA_1 contributes to osteoclast formation in vitro. **A–D,** Bone marrow cells derived from LPA_1-deficient and WT mice were cultured with macrophage colony-stimulating factor (M-CSF) (10 ng/ml) for 2 days, and further cultured with RANKL (70 ng/ml) and M-CSF (10 ng/ml) for 3 days. **E–H,** Bone marrow cells derived from WT mice were cultured with M-CSF (10 ng/ml) for 2 days, and further cultured with RANKL (70 ng/ml) and M-CSF (10 ng/ml) in the presence or absence of the LPA_1 antagonist LA-01 (10 n*M*) for 3 days. **A, B, E,** and **F,** Cultured cells were stained with tartrate-resistant acid phosphatase (TRAP) (**A** and **E**), and TRAP-positive multinucleated cells (MNCs) (≥3 nuclei) were counted. **C, D, G,** and **H,** Resorptive activity was assessed by cell culture on calcium phosphate–coated plates (**C** and **G**), and the area of resorption lacunae was examined under light microscopy (**D** and **H**). **Arrows** in **C** and **G** indicate resorption lacunae. Values in **B, D, F,** and **H** are the mean ± SEM. $* = P < 0.05$. Bars in **A, C, E,** and **G** = 100 μm. See Figure 2 for other definitions.

deficient mice into the synovium of mice with CIA was significantly decreased compared with migration of CD11b+ splenocytes from WT mice (Figures 4A and B). We also analyzed the effect of LPA_1 antagonist on cell infiltration. LA-01 was administered at 12 hours before and 30 minutes before the transfer and 12 hours after the transfer. This short-term treatment of recipient mice with CIA did not alter the arthritis score. The same treatment regimen did, however, reduce the infiltration of CD11b+ cells into the arthritic joints of WT mice (Figures 4C and D). These results indicate that LPA_1 plays an important role in the migration of macrophages into inflamed synovium.

Inhibition of differentiation into Th17 in vitro by LPA_1 deficiency. Previous studies have identified a role of Th17 in the development of arthritis (32,33) and, as described above, we demonstrated reduced in vivo IL-17 production by CII-stimulated splenocytes from CII-immunized mice that were deficient in LPA_1 or treated with an LPA_1 inhibitor. Therefore, we analyzed the effect of LPA_1 on T helper cell differentiation in vitro. CD4+ naive T cells from LPA_1-deficient or WT mice were incubated with anti-CD3 and anti-CD28 mAb under Th1-, Th2-, or Th17-polarizing conditions. Under Th1-polarizing conditions, the proportion of IFNγ-producing cells and the expression level of mRNA for T-bet, a transcription factor for Th1 development, were not significantly different between CD4+ T cells from LPA_1-deficient and WT mice (Figures 5A and B). The proportion of IL-4–producing cells and the expression of GATA-3, a transcription factor in Th2 development, were also not significantly different in cells from LPA_1-deficient mice compared with those from WT mice under Th2-polarizing conditions.

Interestingly, when the cells were incubated under Th17-polarizing conditions, the proportion of IL-17—producing cells and expression of mRNA for RORγt, a transcription factor for Th17 differentiation, were significantly suppressed in LPA_1-deficient mice compared with WT mice (Figures 5A and B). Incubation with LA-01 also reduced the proportion of IL-17–producing cells and RORγt mRNA expression on the cells from WT mice under Th17-polarizing conditions (Figures 5C and D). The proportion of IFNγ-producing cells and IL-4–producing cells and expression of mRNA for T-bet and GATA-3 were not significantly altered following incubation with LPA_1 antagonist under Th1-polarizing and Th2-polarizing conditions. These results indicate that LPA_1 contributes to the differentiation of T cells into Th17.

Role of LPA_1 in osteoclast formation in vitro. To analyze the effect of LPA_1 on osteoclastogenesis, we evaluated in vitro osteoclast formation and calcium phosphate resorption, in BMMs from LPA_1-deficient and WT mice. BMMs were cultured with RANKL and M-CSF. Under these culture conditions, WT mouse BMMs transformed into TRAP-positive multinucleated cells (osteoclasts). In contrast, the number of TRAP-

positive multinucleated cells from LPA_1-deficient mouse BMMs was significantly lower (Figures 6A and B). Of note, few resorption areas of calcium phosphate were detected in LPA_1-deficient mice (Figures 6C and D). In addition, LA-01 inhibited osteoclast formation from BMMs of WT mice (Figures 6E-H). These results suggest that LPA/LPA_1 signaling plays an important role in osteoclastogenesis as well.

DISCUSSION

In this study, we found that LPA_1 was highly expressed in the RA synovium and that abrogation of LPA_1 ameliorated murine CIA, with less inflammatory cell infiltration, bone destruction in the joints, and IL-17 production by CII-stimulated splenocytes. Our results also demonstrated a role of LPA/LPA_1 signaling in macrophage migration, Th17 differentiation, and osteoclastogenesis. These findings highlight the potential participation of LPA_1 in the development of arthritis.

Recent studies demonstrated that ATX was expressed on FLS and in the ST and SF of patients with RA, and that conditional genetic ablation of ATX in mesenchymal cells resulted in disease attenuation in animal models of arthritis (18). It has been suggested that the ATX/LPA axis plays an important role in the development of arthritis. However, the contribution of individual LPA receptors to the arthritis has not been elucidated. Blockade of ATX results in reduction of LPA, and signaling from all LPA receptors (LPA_{1-6}) should be decreased. In addition, ATX-deficient mice are recessive embryonic lethal and have exhibited severe vascular defects (19) and neurologic damage (34). Thus, ATX may not be an appropriate target molecule for the treatment of arthritis, and there is a need to identify an individual LPA receptor that is important in the development of arthritis, which could be a specific target in a novel strategy for RA therapy. In this study, we have demonstrated key roles of LPA_1 in inflammatory cell migration, Th17 differentiation, and osteoclast formation. Furthermore, LPA_1-deficient mice did not develop CIA.

CD68+ macrophages are abundant in RA synovium. They produce inflammatory cytokines, such as TNF and IL-6, which contribute to the pathogenesis of RA (1). We have shown in the present study that RA synovial macrophages expressed LPA_1, and that infiltration of transferred macrophages from LPA_1-deficient mice into the synovium of mice with CIA was significantly suppressed compared with WT mouse macrophages; LPA_1 antagonist blocked the migration of WT mouse macrophages. These results suggest that LPA, like chemokines, is an important chemoattractant for synovial inflammation (29), and that LPA_1 is responsible for this migration.

Th17 cells are a distinct T cell subset involved in autoimmune disease (32,33,35–37). IL-17 enhances production of inflammatory cytokines and chemokines from RA synoviocytes (38). Furthermore, deficiency of IL-17 is reported to inhibit murine CIA (33). Our in vitro studies showed that LPA_1-deficient mouse T cells and LPA_1 blockade significantly reduced differentiation into Th17 and expression of RORγt, a key transcriptional molecule for Th17 differentiation. These results indicate the importance of LPA_1 in Th17 differentiation. IL-17 production by CII-stimulated splenocytes from LPA_1-deficient mice or LPA_1 antagonist–treated mice with CIA was decreased, which suggests that LPA_1 might also play a pivotal role in Th17 differentiation in vivo in mice with CIA. In addition, IL-17 mRNA levels in the synovium of mice with CIA were reduced by the treatment with LA-01. The reduced differentiation of Th17 cells might also contribute to the attenuation of arthritis. However, we were not able to analyze LPA receptor expression on Th1, Th2, and Th17 cells. Further studies are needed to compare the expression of LPA receptors between T helper cells.

Bone destruction observed in RA joints, which is caused by activation of osteoclasts, leads to articular dysfunction. Previous studies have shown that stimulation with LPA induced osteoclast fusion and was required for formation of multinucleated osteoclasts (39). Therefore, it was suggested that LPA may also play a crucial role in osteoclast formation. In this study, we demonstrated that abrogation of LPA_1 diminished osteoclast formation, suggesting that LPA_1 is likely the responsible receptor for LPA-induced osteoclastogenesis. Moreover, blockade of LPA_1 reduced bone destruction and numbers of TRAP-positive osteoclasts in the joints of mice with CIA. Thus, LPA_1 antagonist treatment might be useful for inhibition of bone destruction in RA. It has also been reported that LPA_1-deficient mice had altered bone metabolism and that LPA_1 plays a role in bone metastasis of malignant cells (40,41), consistent with its involvement in osteoclastogenesis.

In the present study, we have demonstrated important roles of LPA_1 in the development of arthritis. Although LPA_{2-6} were also expressed in RA synovium, their effects on the arthritis remain unclear. Further studies are needed to scrutinize the roles of LPA_{2-6} in the pathogenesis of RA. In conclusion, our findings strongly suggest that inhibition of LPA_1 signaling ameliorates arthritis due to inhibition of inflammatory cell migration, Th17 differ-

entiation, and osteoclastogenesis. LPA_1 could thus be a promising therapeutic target for RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Nanki had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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