

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 LPA₁ 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 LPA₁ on collagen-induced arthritis (CIA) were evaluated using LPA₁-deficient mice or LPA₁ 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. LPA₁ was highly expressed in the synovium of patients with rheumatoid arthritis (RA) compared with that of patients with osteoarthritis. LPA₁-deficient mice did not develop arthritis following immunization with type II collagen (CII). LPA₁ antagonist also ameliorated murine CIA. Abrogation of LPA₁ 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 LPA₁-deficient mice into the synovium was suppressed compared with infiltration of macrophages from wild-type mice. LPA₁ 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 LPA₁ deficiency or LPA₁ inhibition in vitro.

Conclusion. Collectively, these results indicate that LPA/LPA₁ signaling contributes to the development of arthritis via cellular infiltration, Th17 differentiation, and osteoclastogenesis. Thus, LPA₁ 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 LPA₁ have been shown to have decreased fibroblast recruitment and vascular leak, and LPA₁ 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 LPA₁ in inflammatory cell infiltration, Th17 differentiation, and osteoclastogenesis and showed that abrogation of LPA₁ ameliorated arthritis, suggesting that LPA₁ 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 \pm 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- μ m-thick sections) from RA and OA patients and from mice with collagen-induced arthritis (CIA) (see below) were deparaffinized and then immersed in 1 mM 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% H₂O₂ for 30 minutes. Sections were then blocked with 1% skim milk for 45 minutes and stained with rabbit anti-ATX polyclonal antibody (2 μ g/ml; Cayman Chemical), anti-LPA₁ polyclonal antibody (10 μ g/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 LPA₁, 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 μ g/ml; DakoCytomation) or anti-vWF mAb (F8/86) (8 μ g/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-LPA₁ polyclonal antibody, or isotype control as described above for 45 minutes at room temperature, and then with Alexa Fluor 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 LPA₁ (sense 5'-ACC-CAA-TAC-TCG-GAG-ACT-GAC-TGT-3', antisense 5'-CGT-CAG-GCT-GGT-GTC-AAT-GA-3'), LPA₂ (sense 5'-TCA-TCA-TGG-GCC-AGT-GCT-ACT-3', antisense 5'-GTG-GGA-GCT-GAG-CTC-TTT-GC-3'), LPA₃ (sense 5'-CTT-GAC-TGC-TTC-CCT-CAC-CAA-3', antisense 5'-CGC-ATC-CTC-ATG-ATT-GAC-ATG-3'), LPA₄ (sense 5'-TCC-TCA-GTG-GCG-GTA-TTT-CAG-3', antisense 5'-AAG-CAG-GTG-GTG-GTT-GCA-TT-3'), LPA₅ (sense 5'-GGT-GGT-

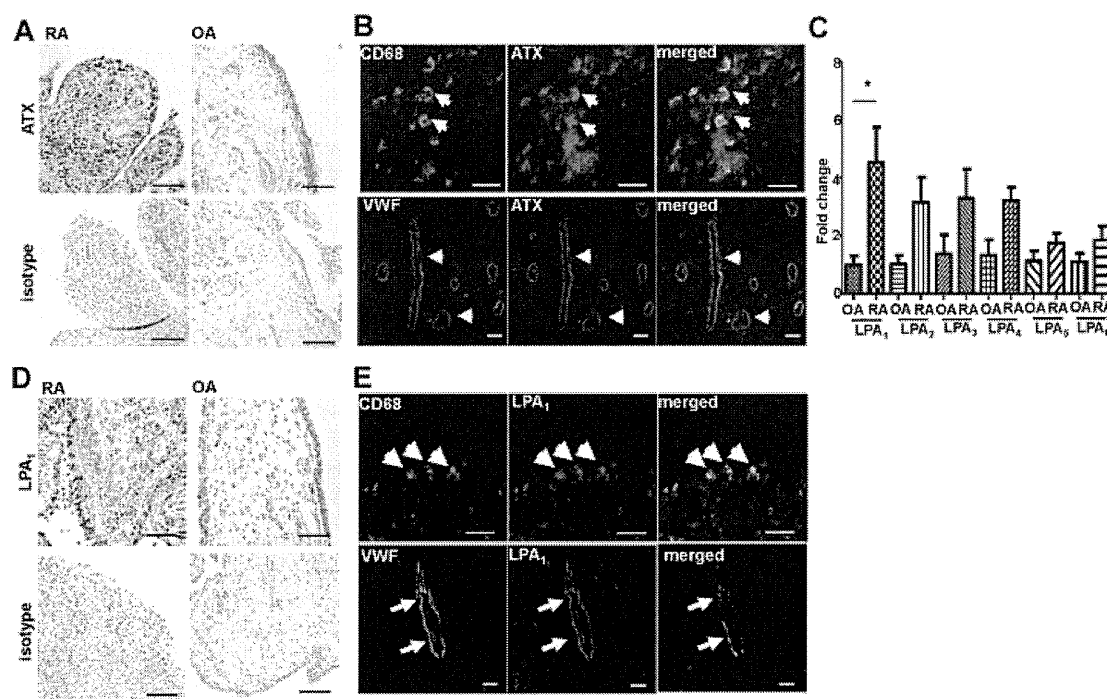


Figure 1. Increased expression of autotaxin (ATX) and lysophosphatidic acid receptor 1 (LPA₁) in rheumatoid arthritis (RA) synovium. **A**, Expression of ATX in the synovium of RA patients ($n = 5$) and osteoarthritis (OA) patients ($n = 5$) was analyzed by immunohistochemistry. **B**, RA synovium was double-stained with CD68 or von Willebrand factor (vWF) and ATX. **Arrows** and **arrowheads** indicate double-positive cells. **C**, Expression levels of LPA₁₋₆ 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 \pm SEM. * = $P < 0.05$. **D**, Expression of LPA₁ 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 LPA₁. **Arrows** and **arrowheads** indicate double-positive cells. Bars in **A** and **D** = 100 μ m; bars in **B** and **E** = 50 μ m. Representative images are shown.

GAG-CGT-GTA-CAT-GTG-T-3', antisense 5'-AGT-GGT-GCA-GTG-CGT-AG-TAG-GA-3'), LPA₆ (sense 5'-AGA-ACC-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 LPA₁-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- μ m-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 LPA₁ antagonist. DBA1/J mice with CIA were administered an LPA₁ 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

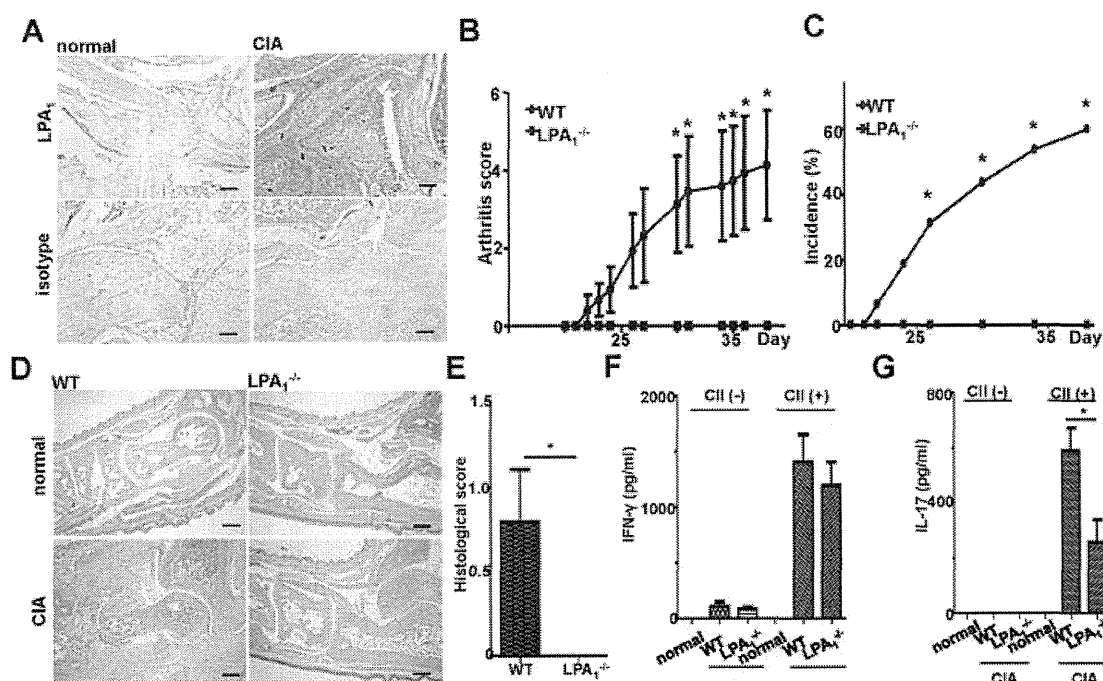


Figure 2. Inhibition of arthritis development in lysophosphatidic acid receptor 1 (LPA₁)-deficient mice. Collagen-induced arthritis (CIA) was induced in LPA₁-deficient mice on a C57BL/6 background ($n = 17$) and in wild-type (WT) mice ($n = 16$) by immunization with chicken type II collagen (CII) on days 0 and 21. **A**, Expression of LPA₁ in the synovium of CII-immunized and normal mice, analyzed by immunohistochemistry. **B** and **C**, Clinical arthritis score (**B**) and arthritis incidence (**C**) in LPA₁-deficient and WT mice. **D**, Histologic features in hematoxylin and eosin-stained ankle joints of normal and CII-immunized LPA₁-deficient and WT mice. **E**, Histologic scores in LPA₁-deficient and WT mice. **F** and **G**, Concentrations of interferon- γ (IFN γ) (**F**) and interleukin-17 (IL-17) (**G**) in the culture supernatants of CII-stimulated splenocytes from LPA₁-deficient and WT mice, determined by enzyme-linked immunosorbent assay. Values in **B**, **E**, **F**, and **G** are the mean \pm SEM. * = $P < 0.05$. Bars in **A** and **D** = 300 μ m. Representative images are shown.

arthritis were assessed. LPA₁, LPA₂, and LPA₃ 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 LPA₁ receptor antagonist (50% inhibition concentration 0.086 μ moles/liter, 2.8 μ moles/liter, and 0.90 μ moles/liter for LPA₁, LPA₂, and LPA₃, respectively). LPAs 4–6 are classified as non-Edg family LPA receptors and are structurally distant from the Edg family LPA receptors (28). LPA₄, LPA₅, and LPA₆, respectively, share only 22%, 23%, and 22% amino acid homology with LPA₁.

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 μ g/ml denatured (100°C, 10 minutes) CII (23). Seventy-two hours later, concentrations of interferon- γ (IFN γ) 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 μ g/ml) and anti-CD28 mAb (2 μ g/ml) (both from eBioscience), with or without LA-01 (10 nM), 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 μ g/ml) (BioLegend) and anti-IFN γ mAb (XMGI.2) (10 μ g/ml) (eBioscience); for Th1, anti-IL-4 mAb

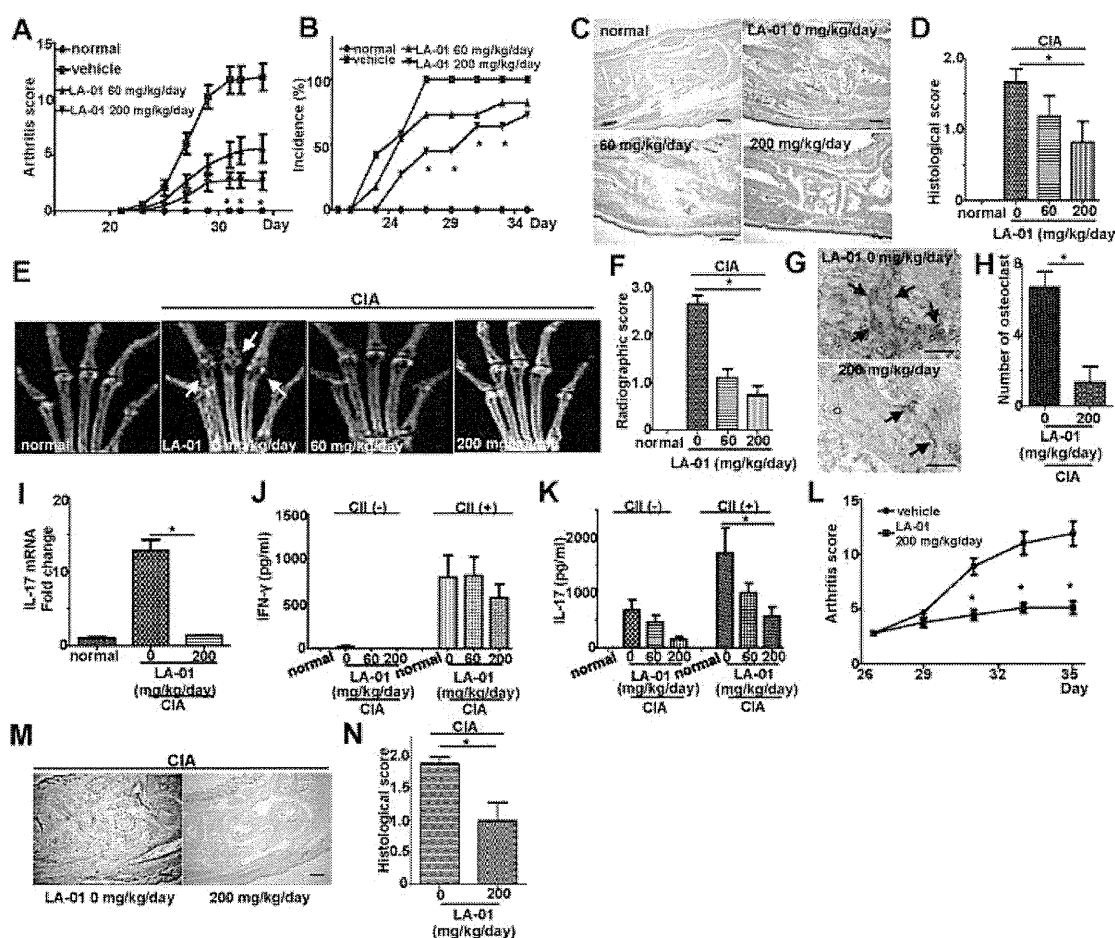


Figure 3. Amelioration of CIA by the LPA₁ antagonist LA-01. DBA1/J mice were immunized with bovine CII on days 0 and 21 and LA-01 was administered twice daily from day 21 to day 35 (A–K) or, in experiments to determine its therapeutic effects, was administered from day 27 to day 35 to mice with established 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 IFN- γ (**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 \pm SEM. * = $P = 0.05$. Bars in **C** and **M** = 300 μ m; bars in **G** = 600 μ m. Representative images are shown. See Figure 2 for other definitions.

(10 μ g/ml) and IL-12 (10 ng/ml) (PeproTech); for Th2, anti-IFN- γ mAb (10 μ g/ml) and IL-4 (10 ng/ml); for Th17, anti-IFN- γ mAb (10 μ g/ml), anti-IL-4 mAb (10 μ g/ml), and IL-6 (30 ng/ml), transforming growth factor β (2.5 ng/ml), IL-21 (80 ng/ml), IL-1 α (10 ng/ml), and TNF α (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 μ g/ml; Sigma-Aldrich) in the presence of

GolgiStop (BD Biosciences) for 4 hours before intracellular staining. For intracellular cytokine staining, allophycocyanin-conjugated anti-mouse IFN- γ 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 γ (ROR γ t) 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 ROR γ t (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 nM) (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 (Bio-coat 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 \pm 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.

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 \pm 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 LPA₁ mRNA was significantly higher in RA ST than in OA ST (Figure 1C). Immunohistochemistry analysis revealed LPA₁ expression on synovial lining and sublining cells, and this expression was also higher compared with OA synovium (Figure 1D). Double staining showed that LPA₁ was expressed on stromal cells, including CD68+ macrophages and vWF-positive endothelial cells in the RA synovium (Figure 1E).

Necessity of LPA₁ for development of CIA in mice. Expression of LPA₁ was markedly increased in the synovium of mice with CIA compared with normal

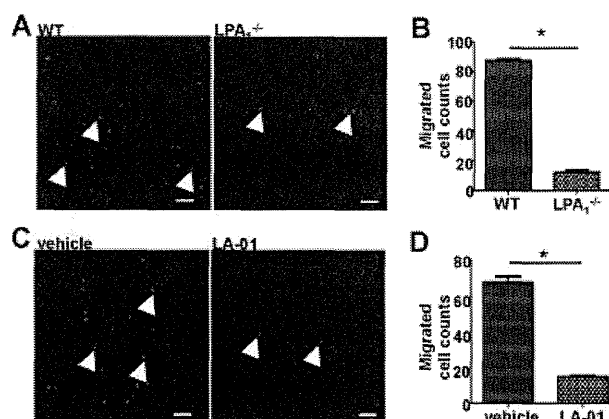


Figure 4. Inhibition of CD11b+ splenocyte migration into the synovium by abrogation of LPA₁. **A** and **B**, Fluorescence-labeled CD11b+ splenocytes from LPA₁-deficient and WT mice were transferred into WT mice with CIA on day 25, and 24 hours after transfer the number of labeled cells that migrated 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 LPA₁ 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 \pm SEM. * = *P* < 0.05. Bars in **A** and **C** = 50 μ m. Representative images are shown. See Figure 2 for definitions.

synovium (Figure 2A). To examine the role of LPA₁ in arthritis, we evaluated CIA in LPA₁-deficient mice. Interestingly, mice lacking LPA₁ 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 LPA₁-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 IFN γ and IL-17 by CII-stimulated splenocytes from mice with CIA. Production of IFN γ and IL-17 was increased in splenocytes from WT mice upon CII stimulation. CII-induced production of IL-17 was decreased in splenocytes from LPA₁-deficient mice compared to that in splenocytes from WT mice, whereas the difference in CII-induced IFN γ 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-

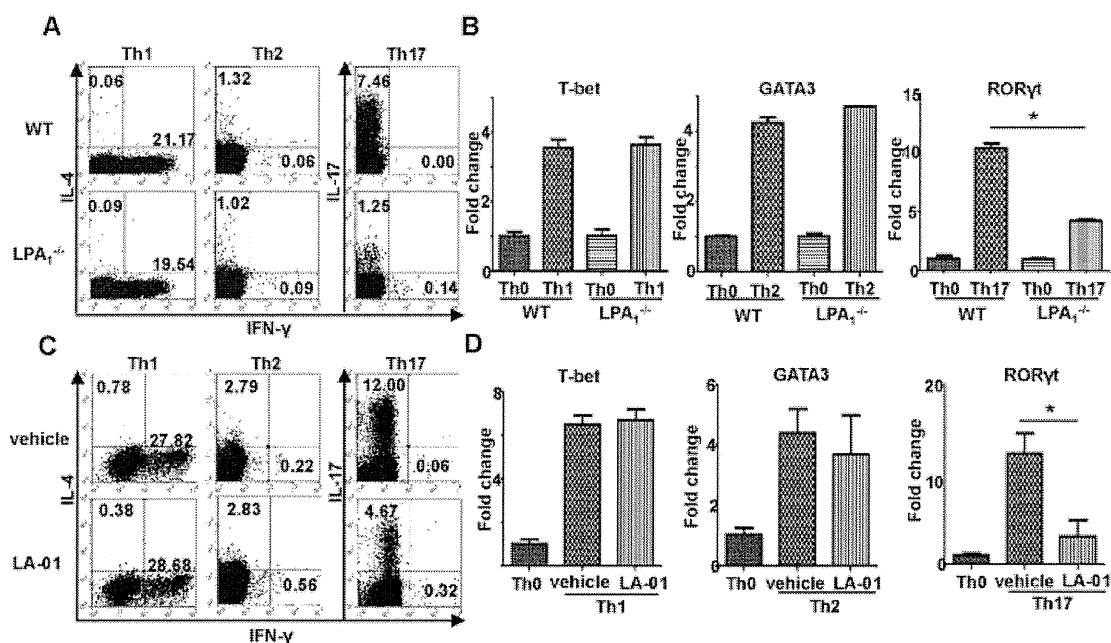


Figure 5. Deficiency of LPA₁ inhibits Th17 differentiation in vitro. **A** and **B**, CD4⁺ T cells from naive LPA₁-deficient and WT mice were cultured with anti-CD3 and anti-CD28 monoclonal antibodies (mAb) under Th1-, Th2-, or Th17-polarizing conditions. Proportions of IFN γ -, IL-4-, and IL-17-producing cells were analyzed by flow cytometry (**A**), and expression of mRNA for T-bet, GATA-3, and retinoic acid receptor-related orphan nuclear receptor γ t (ROR γ t) 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 LPA₁ antagonist LA-01 (10 nM) under Th1-, Th2-, or Th17-polarizing conditions. Proportions of IFN γ -, 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 ROR γ t was quantified by RT-PCR (**D**). Values in **B** and **D** are the mean \pm SEM. * = $P < 0.05$. See Figure 2 for other definitions.

cantly different between LPA₁-deficient and WT mice (data not shown).

We next analyzed the effect of LPA₁ 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 IFN γ , by CII-stimulated splenocytes from mice with CIA (Figures 3J and K), similar to findings in LPA₁-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 LPA₁ 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 LPA₁ contributes to inflammatory cell infiltration, bone erosion, and IL-17 production in vivo. Collectively, they suggest that the LPA/LPA₁ cascade might play an important role in the development of arthritis.

Inhibition of inflammatory cell infiltration by abrogation of LPA₁. To investigate the role of LPA₁ in inflammatory cell infiltration into the synovium, fluorescence-labeled CD11b⁺ splenocytes from LPA₁-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 LPA₁-

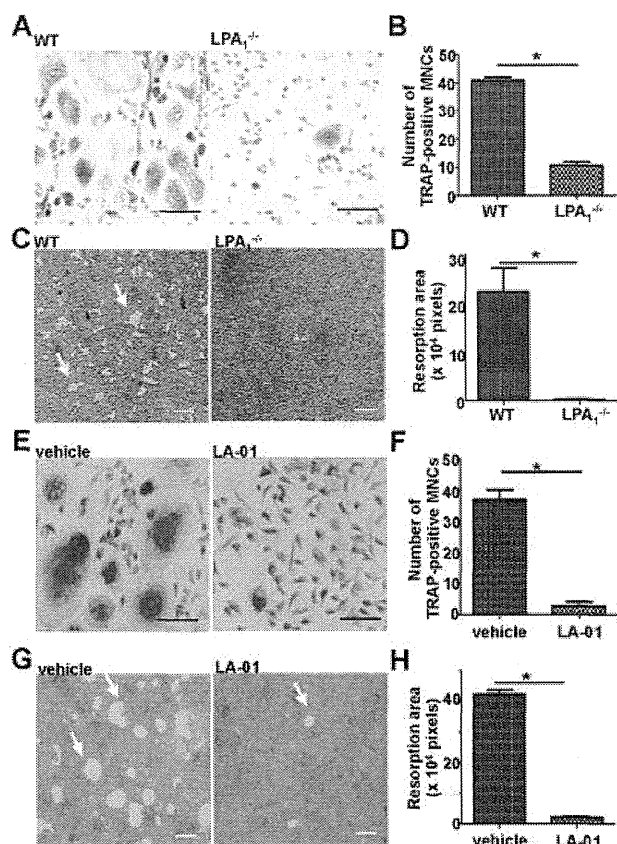


Figure 6. LPA₁ contributes to osteoclast formation in vitro. **A–D**, Bone marrow cells derived from LPA₁-deficient and WT mice were cultured with macrophage colony-stimulating factor (M-CSF) (10 ng/ml) for 2 days, and further cultured with RANKL (70 ng/ml) and M-CSF (10 ng/ml) for 3 days. **E–H**, Bone 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 LPA₁ antagonist LA-01 (10 nM) 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 \pm SEM. * = $P < 0.05$. Bars in **A, C, E, and G** = 100 μ m. 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 LPA₁ 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 LPA₁ plays an important role in the migration of macrophages into inflamed synovium.

Inhibition of differentiation into Th17 in vitro by LPA₁ 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 LPA₁ or treated with an LPA₁ inhibitor. Therefore, we analyzed the effect of LPA₁ on T helper cell differentiation in vitro. CD4+ naive T cells from LPA₁-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 IFN γ -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 LPA₁-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 LPA₁-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 ROR γ t, a transcription factor for Th17 differentiation, were significantly suppressed in LPA₁-deficient mice compared with WT mice (Figures 5A and B). Incubation with LA-01 also reduced the proportion of IL-17-producing cells and ROR γ t mRNA expression on the cells from WT mice under Th17-polarizing conditions (Figures 5C and D). The proportion of IFN γ -producing cells and IL-4-producing cells and expression of mRNA for T-bet and GATA-3 were not significantly altered following incubation with LPA₁ antagonist under Th1-polarizing and Th2-polarizing conditions. These results indicate that LPA₁ contributes to the differentiation of T cells into Th17.

Role of LPA₁ in osteoclast formation in vitro. To analyze the effect of LPA₁ on osteoclastogenesis, we evaluated in vitro osteoclast formation and calcium phosphate resorption, in BMMs from LPA₁-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 LPA₁-deficient mouse BMMs was significantly lower (Figures 6A and B). Of note, few resorption areas of calcium phosphate were detected in LPA₁-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/LPA₁ signaling plays an important role in osteoclastogenesis as well.

DISCUSSION

In this study, we found that LPA₁ was highly expressed in the RA synovium and that abrogation of LPA₁ 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/LPA₁ signaling in macrophage migration, Th17 differentiation, and osteoclastogenesis. These findings highlight the potential participation of LPA₁ 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 (LPA₁₋₆) 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 LPA₁ in inflammatory cell migration, Th17 differentiation, and osteoclast formation. Furthermore, LPA₁-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 LPA₁, and that infiltration of transferred macrophages from LPA₁-deficient mice into the synovium of mice with CIA was significantly suppressed compared with WT mouse macrophages; LPA₁ 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 LPA₁ 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 LPA₁-deficient mouse T cells and LPA₁ blockade significantly reduced differentiation into Th17 and expression of ROR γ t, a key transcriptional molecule for Th17 differentiation. These results indicate the importance of LPA₁ in Th17 differentiation. IL-17 production by CII-stimulated splenocytes from LPA₁-deficient mice or LPA₁ antagonist-treated mice with CIA was decreased, which suggests that LPA₁ 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 LPA₁ diminished osteoclast formation, suggesting that LPA₁ is likely the responsible receptor for LPA-induced osteoclastogenesis. Moreover, blockade of LPA₁ reduced bone destruction and numbers of TRAP-positive osteoclasts in the joints of mice with CIA. Thus, LPA₁ antagonist treatment might be useful for inhibition of bone destruction in RA. It has also been reported that LPA₁-deficient mice had altered bone metabolism and that LPA₁ 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 LPA₁ in the development of arthritis. Although LPA₂₋₆ were also expressed in RA synovium, their effects on the arthritis remain unclear. Further studies are needed to scrutinize the roles of LPA₂₋₆ in the pathogenesis of RA. In conclusion, our findings strongly suggest that inhibition of LPA₁ signaling ameliorates arthritis due to inhibition of inflammatory cell migration, Th17 differ-

entiation, and osteoclastogenesis. LPA₁ 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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Acquisition of data. Y. Miyabe, C. Miyabe, Iwai, Takayasu, Yokoyama, Nagai, Jona, Tokuhara, Ohkawa, Albers, Ovaa, Chun, Ueda, Nanki.

Analysis and interpretation of data. Y. Miyabe, Fukuda, Albers, Yatomi, M. Miyasaka, N. Miyasaka, Nanki.

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CCL18 Activates Fibroblast-like Synoviocytes in Patients with Rheumatoid Arthritis

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CCL18 Activates Fibroblast-like Synoviocytes in Patients with Rheumatoid Arthritis

To the Editor:

Fibroblast-like synoviocytes (FLS) proliferate in the synovial tissue of patients with rheumatoid arthritis (RA), and contribute to chronic inflammation and the destruction of articular cartilage due to the production of a variety of cytokines, chemokines, and matrix metalloproteinases (MMP)¹. The expression of CC chemokine ligand 18 (CCL18) was shown to be increased in the RA synovium^{2,3}; however, the pathogenic role of CCL18 remains unclear. In this study, we investigated the expression of a recently identified receptor for CCL18, phosphatidylinositol transfer membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3)⁴ in the RA synovium, and the stimulatory effects of CCL18 on FLS.

Synovial tissue samples were obtained from patients with RA (n = 4) and osteoarthritis (OA) (n = 4) undergoing joint replacement surgery. RA patients were a median 65 years old (range 48–85 yrs), with median disease duration 9 years (range 2.5–30 yrs) and median C-reactive protein level 1.23 mg/dl (range 0.68–2.85). All RA patients were positive for rheumatoid factor and anticitrullinated protein antibodies. All subjects provided informed consent. The experimental protocol was approved in advance by the Ethics Committee of Tokyo Medical and Dental University.

CCL18-positive cells were observed in the synovial lining, sublining, and perivascular regions of the RA synovium (Figure 1A). CCL18 expression was minimal in the OA synovium (Figure 1C). Western blotting analysis showed that CCL18 expression was significantly higher in the RA synovium than in the OA synovium (Figure 1E, 1F). Double-immunofluorescence staining revealed that most CD68-positive macrophages expressed CCL18 (Figures 1G–1I), and von Willebrand factor (vWF)-positive vascular endothelial cells were also positive for CCL18 (Figures 1J–1L). These results indicated that CCL18 expression was increased in the RA synovium and macrophages were the source, which is consistent with previous studies^{2,3}. We also found that endothelial cells expressed CCL18 in RA synovial tissue.

We then showed the expression of PITPNM3, a receptor for CCL18, in the synovial lining, sublining, and follicle-like aggregates of RA synovial tissue (Figure 1M). PITPNM3 expression was minimal in the OA synovium (Figure 1O). Increased PITPNM3 expression in the RA synovium was confirmed by Western blotting (Figure 1Q, 1R). Double-staining showed that CD68-positive macrophages and FLS (vimentin-positive fibroblast-like appearance cells) expressed PITPNM3 (Figures 1S–1X), while vWF-positive endothelial cells did not (data not shown).

The expression of PITPNM3 in *in vitro* cultured FLS established from RA and OA synovial tissues⁵ was also analyzed by Western blotting. PITPNM3 expression in RA FLS was significantly higher than that in OA FLS (Figure 2A, 2B). To analyze the pathogenic role of CCL18 in RA, we examined the stimulatory effect of CCL18 on RA FLS. FLS established from RA synovial tissue were incubated with CCL18. The production of interleukin 6 (IL-6), CCL2, and MMP-3 was significantly enhanced by stimulation with CCL18 in a dose-dependent manner (Figures 2C–2E). We also analyzed the effect of CCL18 on the proliferation of FLS. Cellular proliferation was not induced by incubation with CCL18 (Figure 2F). Incubation with CCL18 did not alter the motility of FLS as analyzed by the scrape motility assay (data not shown).

CCL18 expression was found to be increased in synovial tissue^{2,3}, synovial fluid^{2,6}, and serum in RA, and serum CCL18 levels correlated with disease severity⁷. However, the pathogenic role of CCL18 in RA has not been established, and the receptor for CCL18 has not been identified. In 2011, Chen, *et al* identified PITPNM3 as a functional receptor for CCL18⁴.

In our study, we confirmed that CCL18 was expressed by RA synovial macrophages and also found that endothelial cells expressed CCL18. PITPNM3, a receptor for CCL18, was also highly expressed in the RA synovium by macrophages and FLS. CCL18 enhanced IL-6, CCL2, and

MMP-3 production in RA FLS *in vitro*. IL-6 has a wide range of functions on lymphocytes, hepatocytes, hematopoietic progenitor cells, and fibroblasts, and also plays important roles in autoimmune diseases. Blockade of IL-6 signaling by anti-IL-6 receptor monoclonal antibody is effective in RA. CCL2 may cause monocyte migration into the synovium⁸. MMP-3 is thought to contribute to pannus invasion and cartilage degradation⁹. Collectively, CCL18 released from synovial macrophages and endothelial cells in the RA synovium may activate FLS and is partly involved in the pathogenesis of RA. CCL18 could induce the migration of T cells, B cells, monocytes/macrophages, and dendritic cells¹⁰. Therefore, CCL18 may also influence inflammatory cell accumulation into the RA synovium.

CCL18 has been shown to induce the phosphorylation of proline-rich tyrosine kinase 2, focal adhesion kinase, and Src family kinase via PITPNM3 in cancer cells⁴. Further studies are needed to clarify the important signaling pathways by which CCL18 stimulation of FLS produces inflammatory mediators.

The interaction of CCL18 and PITPNM3 could play a role in the pathogenesis of RA by activating FLS in the synovium.

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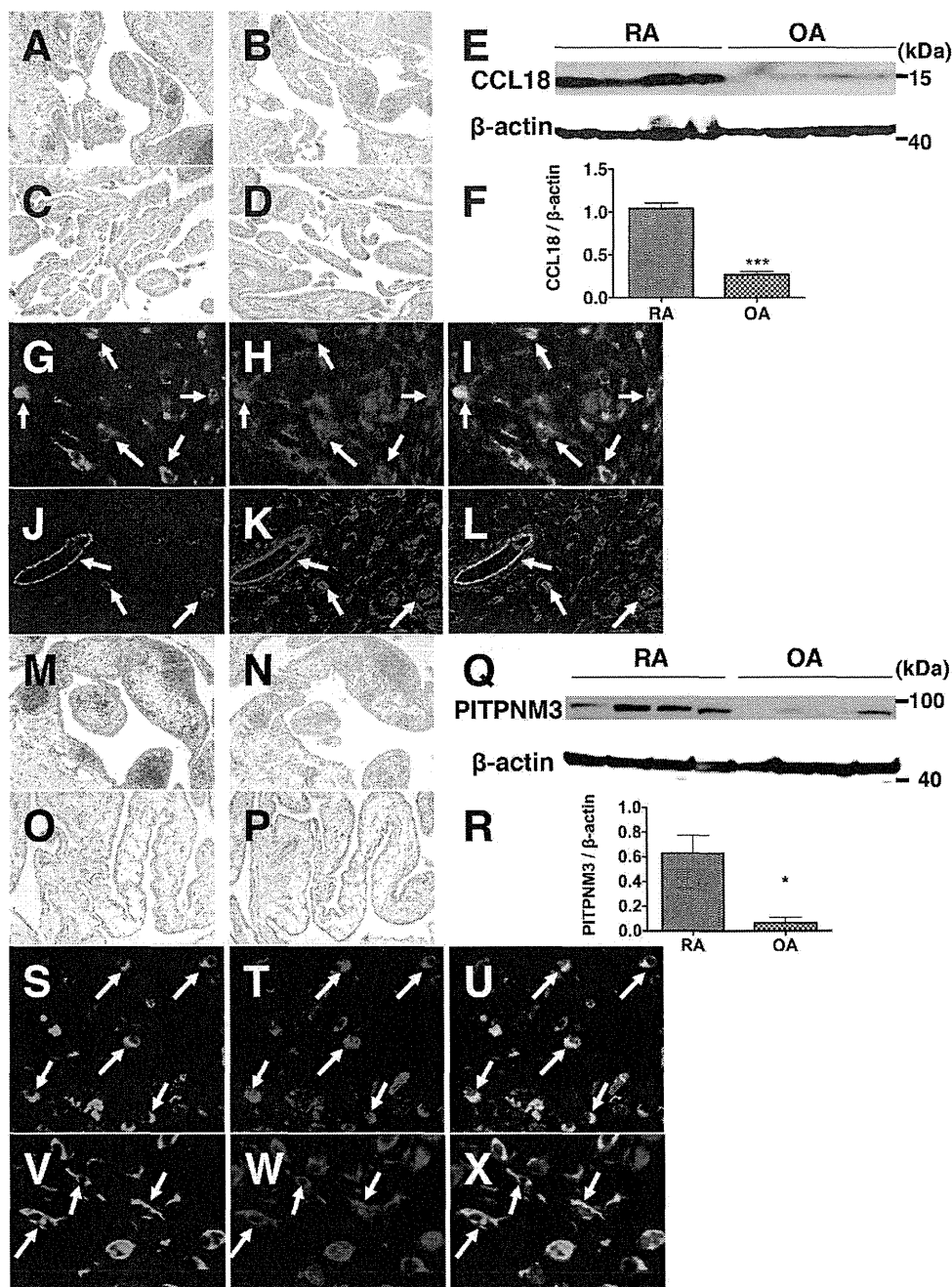


Figure 1. Expression of CCL18 and PITPNM3 in the RA synovium. Synovial tissues were obtained from 4 RA (A, B) and 4 OA patients (C, D), and CCL18 expression was examined by immunohistochemistry. Samples were stained with anti-CCL18 antibody (LifeSpan BioSciences; A, C) or isotype-matched control antibody (B, D). Original magnification $\times 100$. CCL18 expression in RA and OA synovial tissues was also analyzed by Western blotting with anti-CCL18 antibody and anti- β -actin monoclonal antibody (Cell Signaling Technology; E). Protein expression of CCL18 relative to β -actin in RA and OA is shown (F). Values are mean \pm SEM. *** $p < 0.001$, Student t test. Sections of RA synovial tissues were double-stained with anti-CD68 (KP1; DakoCytomation) or -vWF (F8/86; DakoCytomation) monoclonal antibody and anti-CCL18 antibody, and were analyzed by fluorescence microscopy as follows: CD68 (G), CCL18 (H), merged image (I) of (G) and (H), vWF (J), CCL18 (K), merged image (L) of (J) and (K). Arrows indicate double-positive cells. Original magnification $\times 400$ in G to I, $\times 200$ in J to L. Expression of PITPNM3 in RA (M and N) and OA (O and P) synovial tissues was examined by immunohistochemistry. Samples were stained with anti-PITPNM3 antibody (GeneTex; M and O) or isotype-matched control antibody (N and P). Original magnification $\times 100$. PITPNM3 expression in RA and OA synovial tissues was determined by Western blotting (Q). Relative expression of PITPNM3 to β -actin in RA and OA is shown (R). Values are mean \pm SEM. * $p < 0.05$, Student t test. RA synovial tissues were double-stained with anti-CD68 or vimentin (V9; DakoCytomation) monoclonal antibody, and anti-PITPNM3 antibody. CD68 (S), PITPNM3 (T), merged image (U) of (S) and (T), vimentin (V), PITPNM3 (W), merged image (X) of (V) and (W). Arrows indicate double-positive cells. Original magnification $\times 400$.

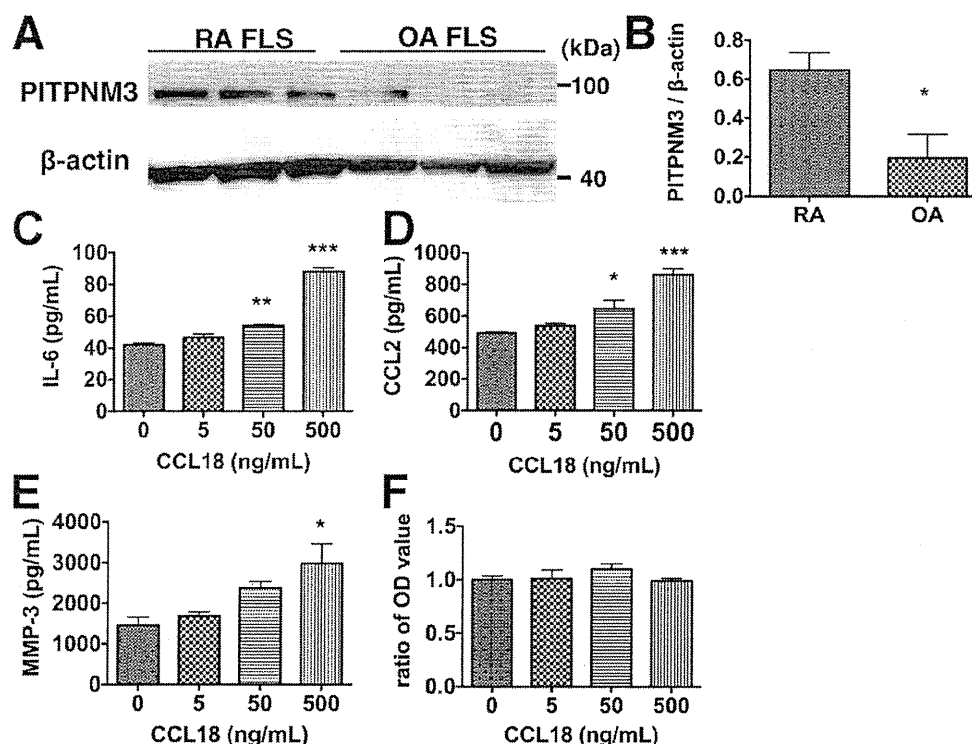


Figure 2. Expression of PITPNM3 in cultured FLS and the stimulatory effect of CCL18. Expression of PITPNM3 in *in vitro* cultured FLS from RA and OA synovial tissues was determined by Western blotting (A). Relative expression of PITPNM3 to β-actin in RA FLS and OA FLS is shown (B). Values are mean ± SEM. **p* < 0.05, Student *t* test. FLS (2×10^3 cells/well) from RA synovial tissue were cultured overnight in a 96-well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Then medium was replaced with serum-free DMEM followed by addition of the indicated concentration of CCL18 (R&D Systems). After 72 hours, IL-6, CCL2, and MMP-3 concentrations in culture supernatant were measured by ELISA (R&D systems) (C: IL-6; D: CCL2; E: MMP-3), and cell proliferation was examined using a cell counting kit (Cell Counting Kit-8; Dojindo; F). Data are presented as means ± SEM of 1 of 3 independent experiments analyzed in triplicate. **p* < 0.05; ***p* < 0.01; ****p* < 0.005 (one-way ANOVA with Dunnett's multiple comparison test).

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LETTER

Suppression of elevations in serum C reactive protein levels by anti-IL-6 autoantibodies in two patients with severe bacterial infections

Serum C reactive protein (CRP) is generally elevated by infection. We encountered two patients with severe bacterial infections without increases in CRP. Patient 1: A 67-year-old man developed thoracic empyema by *Escherichia coli* and *Streptococcus intermedius*. His body

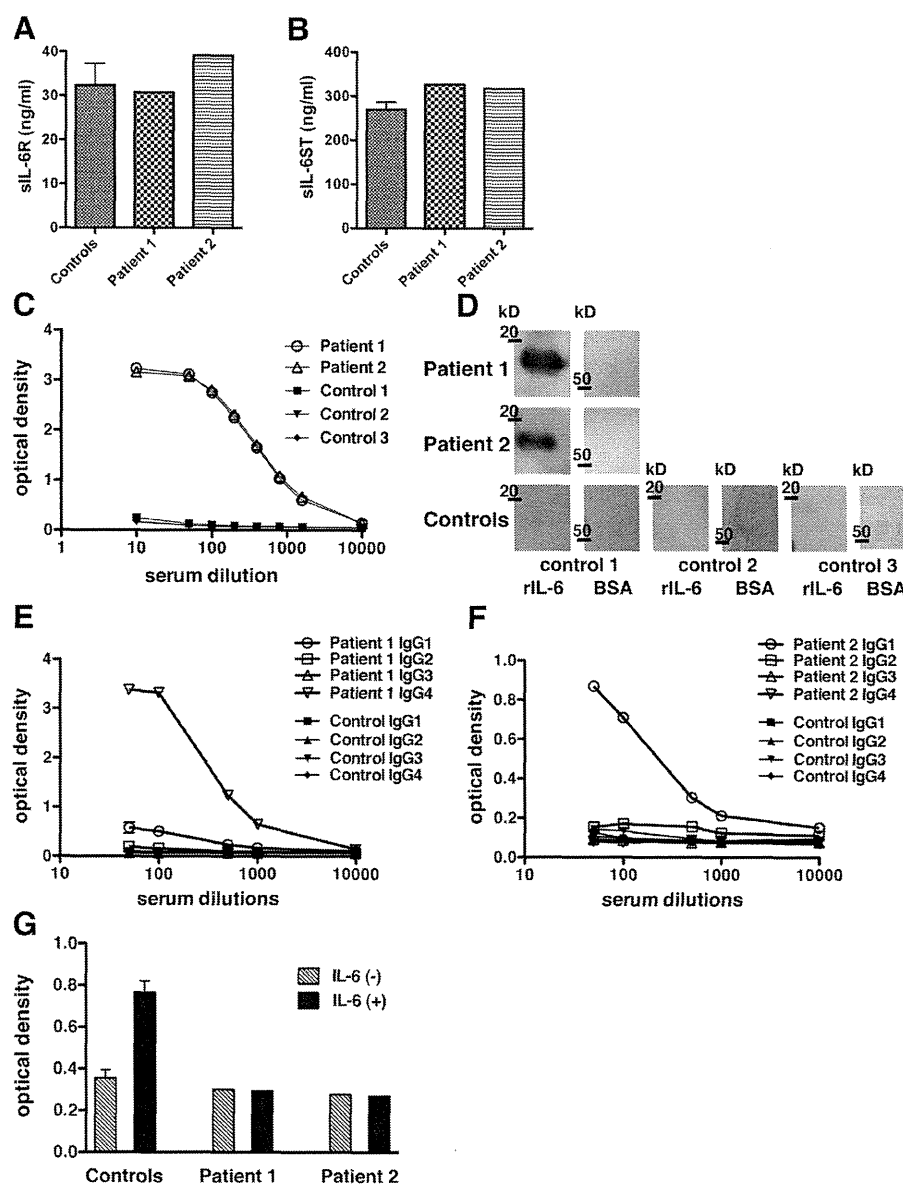
temperature was 36.6°C, his leukocyte count was elevated (9960/μl) and he was negative for CRP (0.01 mg/dl). Despite antibiotic treatment, he died of respiratory failure. Patient 2: A 56-year-old woman developed multiple subcutaneous abscesses by *Staphylococcus aureus*. She had rheumatoid arthritis (RA) for 30 years and was treated with sodium aurothiomalate. Her body temperature was 37.4°C, and leukocyte count (5600/μl) and CRP (0.05 mg/dl) were not elevated. She recovered with antibiotics. Neither patient had a past history of severe bacterial infection.

Since serum CRP is mainly controlled by interleukin (IL)-6,¹ the lack of IL-6 function was suggested. Serum IL-6 was not detected by ELISA in either patient, although it is

known to increase with severe infection.^{2,3} However, IL-6 production from peripheral blood monocytes with/without lipopolysaccharide stimulation was similar between Patient 1 and healthy controls (data not shown). Soluble IL-6 receptor (IL-6R) and IL-6 signal transducer (IL-6ST) serum levels were also similar between patients and controls (figure 1A,B).

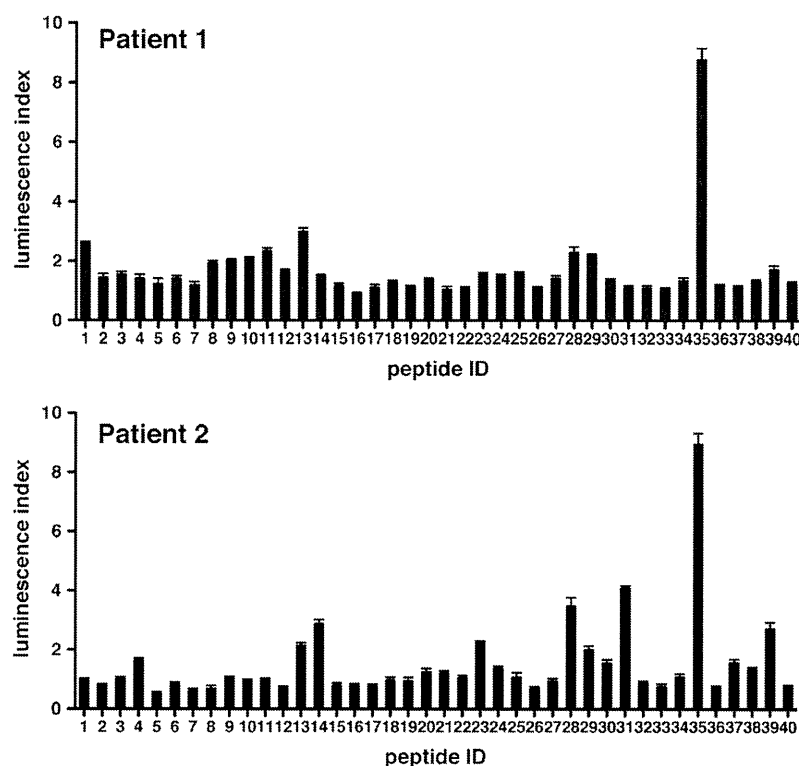
Based on the clinical course and above findings, we hypothesised that patients' sera contain anti-IL-6 antibodies. Figure 1C showed that both patients' sera, but not controls, contained immunoglobulin (Ig)G against IL-6. The presence of anti-IL-6 antibodies was confirmed by western blotting (figure 1D). Isotypes of the autoantibodies of Patients 1 and 2 were IgG4 and IgG1,

Figure 1 Serum sIL-6R and sIL-6ST levels and the detection of anti-IL-6 autoantibodies in the sera of the two patients. Serum sIL-6R (A) and sIL-6ST (B) levels in three healthy controls and in Patients 1 and 2 were measured using ELISA kits (sIL-6R: Life Technologies, Grand Island, New York, USA; sIL-6ST: R&D Systems, Minneapolis, Minnesota, USA). The ELISA plate was coated with recombinant IL-6 (rIL-6), and then diluted serum samples from three healthy controls and Patients 1 and 2 were incubated on these plates. To measure the titre of anti-IL-6 IgG, horseradish peroxidase (HRP)-conjugated mouse antihuman IgG monoclonal antibody (mAb) was added, 3,3', 5,5'-tetramethylbenzidine single solution was reacted, and absorbance at 450 nm was measured (C). Control bovine serum albumin (BSA) and rIL-6 were separated on a sodium dodecyl sulphate-polyacrylamide gel. Proteins were then electrotransferred to a polyvinylidene fluoride microporous membrane in a semidry system. Immunoblots were incubated with serum diluted with phosphate-buffered saline (1:200) from three control subjects and Patients 1 and 2. The membrane was incubated with HRP-conjugated mouse antihuman IgG mAb (D). To determine the isotype of anti-IL-6 IgG from Patients 1 and 2, HRP-conjugated mouse antihuman IgG1, IgG2, IgG3 or IgG4 mAb was used for the ELISA (E: Patient 1; F: Patient 2). TF-1 cells were cultured with rIL-6 (5 ng/ml) in a medium containing 10% serum from three healthy controls, Patient 1 or Patient 2 for 3 days. The number of cells was counted with a cell counting kit and reported as optical density units (G). Control data are mean±SD.



PostScript

Figure 2 Epitope mapping of anti-IL-6 autoantibodies. N-terminal biotinylated 15-mer peptides overlapping by 10 amino acid residues were generated from the human IL-6 protein sequence, and these peptides were coated on ELISA plates. Serum samples from Patients 1 and 2 were added to these plates, and peptide-bound antibodies were detected. Data are presented as the luminescence index (defined as the luminescence signal with the subtracted peptide-specific background, divided by the sample- and dilution-specific background) measured for each peptide/sample combination. Scores for the 1:200 and 1:400 dilutions are presented as mean \pm SD.



respectively (figure 1E,F). The patients' sera suppressed the proliferation of TF-1 cells stimulated with recombinant IL-6 (figure 1G).

These results suggest that anti-IL-6 autoantibodies block IL-6 signalling and inhibit increases in serum CRP. Puel *et al*⁴ reported a young boy with repeated cellulitis at 11 and 29 months of age. Serum CRP was negative and anti-IL-6 autoantibodies were detected. Although this case is similar to our patients, to our knowledge, ours are the first reported adult patients negative for CRP despite severe bacterial infections due to anti-IL-6 autoantibodies. Galle *et al*⁵ detected anti-IL-6 autoantibodies in three of 4230 healthy subjects. Serum CRP in Patient 2 with RA was occasionally positive until 4 years ago, suggesting that anti-IL-6 autoantibodies developed during that time. The infection may have triggered autoantibody production; however, we could not analyse autoantibodies before infection or after recovery.

IL-6 has important immune functions.⁶ Treatment with anti-IL-6R mAb for RA increases the risk of infection such as pneumonia and cellulitis.⁷⁻⁸ Therefore, patients with anti-IL-6 autoantibodies are immunocompromised and special care for infection should be exercised.

We determined the epitope of anti-IL-6 autoantibodies. Both patients' sera strongly bound to peptide 35, LTKLQAQNQWL

QDMT (figure 2), suggesting that this peptide includes the epitope of anti-IL-6 antibodies. This peptide includes a critical amino acid, tryptophan¹⁵⁷, for the binding of IL-6 and IL-6ST to form a dimer of trimers composed of IL-6, IL-6R and IL-6ST.⁹

In conclusion, we detected anti-IL-6 autoantibodies in two patients with severe bacterial infections without elevations in serum CRP.

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Suppression of elevations in serum C reactive protein levels by anti-IL-6 autoantibodies in two patients with severe bacterial infections

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CXCR7 agonists inhibit the function of CXCL12 by down-regulation of CXCR4

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ABSTRACT

The CXCL12/CXCR4 axis is involved in many cellular responses for host homeostasis, and malfunction of this signaling pathway is associated with a variety of diseases. It is now known that CXCL12 also binds to another newly identified chemokine receptor, CXCR7, which does not couple with a G-protein. CXCR7 can form homodimers, or heterodimers with CXCR4, and is believed to sequester the chemokine CXCL12, although the CXCL12/CXCR7 axis activates MAP kinases through β-arrestin. Therefore, it has not been well defined how CXCR7 activation affects CXCL12-induced cellular events. To elucidate the function of CXCR7, we prepared CXCR7 agonist Compound 1. Compound 1 is a selective and potent CXCR7 agonist that clearly has the activity to recruit β-arrestin toward CXCR7. It also activates MAP kinases Akt and ERK. Using this compound, we confirmed that the CXCR7 agonist, but not an antagonistic antibody, did inhibit CXCL12 induced HUVEC tube formation, suggesting that activation of CXCR7 ameliorates CXCL12 induced cellular events, probably by affecting on CXCR4 function. We show that β-arrestin recruitment to CXCR4 is reduced by over-expression of CXCR7 and activation of CXCR7 by agonist treatment reduces the protein level of CXCR4. Based on our results, together with reported information, we propose that CXCR7, when up-regulated upon inflammation, can act as a negative regulator of CXCR4 by heterodimerizing with CXCR4, inducing its internalization and degradation. This mechanism suggests that CXCR7 agonists can have a therapeutic effect on CXCL12 causing diseases by countering the effects of CXCL12.

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1. Introduction

CXCL12, also called SDF-1 (stromal cell-derived factor-1), is a chemokine known as a critical factor in several diseases including cancer or autoimmune diseases. Accumulating evidence has been implicating CXCL12 in tumor cell metastasis and proliferation [1,2]. In the case of Rheumatoid Arthritis (RA), the expression of CXCL12 is upregulated in the synovial tissue of RA patients compared to that of osteoarthritis patients, and CXCL12 may act to induce leukocyte accumulation, stimulate chondrocytes to release matrix metalloproteinase 9, and enhance angiogenesis in the synovium [3–5]. CXCL12 used to be believed to bind only to receptor CXCR4, but recently CXCR7 has been identified as another receptor for CXCL12 [6,7]. CXCR7 binds with high affinity to CXCL12 and also CXCL11 (ITAC; interferon-inducible T cell α chemoattractant). Unlike classical chemokine receptors, CXCR7 signals through

β-arrestin in response to agonists without detectable activation of G-proteins [8,9]. A variety of functions of CXCL12 has been demonstrated as a ligand for CXCR4, but the role of CXCR7 is largely unknown yet. Several reports have suggested that CXCR7 associates with CXCR4 and affects its internalization, or that CXCR7 scavenges CXCL12 resulting in the modulation of CXCR4 activity [10–12] however, the precise mechanism still remains unclear.

Chemical compounds that specifically bind to CXCR7 showed efficacy in several mice models of cancer or autoimmune diseases such as collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [7,5,13]. Although these compounds had been originally thought to be CXCR7 antagonists, several studies have shown that they have agonistic activity in terms of CXCR7 dependent β-arrestin recruitment [14]. It has not yet been established how CXCR7 agonists ameliorate the clinical scores of various mouse disease models. In our study, we show that upon binding to CXCR7, CXCR7 agonists reduced the expression level of CXCR4 which resulted in reduction of the cell's sensitivity against CXCL12. As a result, CXCR7 agonists negatively regulate CXCL12–CXCR4 induced cellular events such as angiogenesis.

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