Suppressing anti-citrullinated protein antibody-induced osteoclastogenesis in rheumatoid arthritis using anti-CD64 and PAD-2 inhibitors

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Abstract

Objective

To evaluate the role of Fcγ receptors (FcγR) and peptidyl arginine deiminase (PAD) in anti-citrullinated protein antibody (ACPA)-induced fibroblast-like synoviocytes (FLSs)-mediated osteoclastogenesis in patients with rheumatoid arthritis (RA).

Methods

FLSs and peripheral blood mononuclear cells were collected from patients with RA. We stimulated RA-FLS with ACPA (100 ng/ml) with and without anti-cluster of differentiation (CD)32a/CD64 (FcγRIIA/FcγRI) antibody and PAD-2/4 inhibitors. Flow cytometry and enzyme-linked immunosorbent assay were also performed. CD14+ monocytes were cultured with receptor activator of nuclear factor kappa beta (RANKL) and macrophage colony-stimulating factor, and ACPA-stimulated RA-FLSs were added. These cells were cultured for 14 days, and osteoclastogenesis was quantified using tartrate-resistant acid phosphatase (TRAP) staining.

Results

ACPA increased RANKL+ and tumour necrotic factor-alpha (TNF-α+) FLS, which decreased dose-dependently by adding 5 and 10 ug/mL anti-CD64 antibody rather than anti-CD32a antibody. In PAD inhibitor experiments, the proportion of RANKL+ and TNF-α+ FLS decreased in 50 μM condition containing PAD-2 inhibitor rather than PAD-4 inhibitor. The co-culture of ACPA-stimulated RA-FLSs and osteoclast precursors increased the TRAP+ multinucleated osteoclast count, which was decreased by anti-CD64 antibody and PAD2 inhibitor.

Conclusion

The present study showed that ACPA increased RANKL and pro-inflammatory cytokine expression in RA-FLSs, and ACPA-activated RA-FLSs could augment osteoclastogenesis. These processes were inhibited by treatment with anti-CD64 antibody and PAD-2 inhibitors. These results show that CD64 and PAD-2-induced pathways may be involved in ACPA-induced FLS activation and osteoclastogenesis in patients with RA. Therefore, regulating the CD64 and PAD-2 pathways may improve RA treatment.

Key words

anti-citrullinated peptide antibody, rheumatoid arthritis, fibroblast-like synoviocyte, osteoclast, FcγR, peptidyl arginine deiminase

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Received on February 17, 2024; accepted in revised form on July 18, 2024.

Funding: this work was supported by a grant from the Basic Science Research Programme through the National Research Foundation of Korea, funded by the Ministry of Education, Science, and Technology, Republic of Korea (NRF-2021R1A2C1010075).

Competing interests: none declared.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune-mediated inflammatory arthritis (1). The global prevalence of RA is 0.46% (2), and its prevalence in Korea ranges from 0.27–1.85% (3). RA pathogenesis involves various innate and adaptive immune cells (1, 4) and autoreactive B cells, which progress to autoantibody-producing plasma cells. RA-specific autoantibodies include rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), and anti-carbamylated protein antibodies (5). Post-translational modifications, including citrullination and carbamylation, modify self-antigens and induce autoimmune responses (5). Peptidylarginine deiminase (PAD)-2 and -4 enzymes mediate the citrullination response in the RA synovium (6, 7).

The classification criteria for RA involves RF/ACPA positivity and titre (8). RA-specific autoantibodies are important in RA diagnosis and prognosis prediction (9, 10). Furthermore, high RF or ACPA titres are well-known poor prognostic factors for RA (9), and recent guidelines recommend switching to biologic disease-modifying antirheumatic drugs when patients with RA fail to achieve initial treatment targets (low disease activity or remission) (10). ACPA is associated with joint destruction (11), ischaemic heart disease (12), and increased risk of fractures in patients with RA (13). Furthermore, ACPA positivity affects the abatacept retention rate in patients with RA (14). Therefore, ACPA presence and titre provide useful clinical information for predicting RA prognosis and treatment decision-making in patients with RA.

The treat-to-target (T2T) of RA achieves remission or low disease activity (10), and this strategy can reduce arthralgia and prevent joint destruction. Joint destruction is mainly mediated by increased matrix metalloproteinase expression and osteoclastogenesis of the juxta-articular space (4, 15). Therefore, preventing extra-articular comorbidities significantly impacts the quality of life and mortality in patients with RA. The risk of osteoporosis and consequent fracture are possible comorbidities in patients with RA (13, 16). Increased osteoclastogenesis in patients with RA is due to the high expression of pro-inflammatory cytokines and receptor activation of nuclear factor kappa B (NF-κB) (RANK) ligands (17, 18). Furthermore, ACPA directly increases osteoclastogenesis (19). The interaction between RANK expressed on osteoclast precursors and RANK ligands (RANKL) is the most crucial signal for mature osteoclast differentiation (17). Fibroblast-like synoviocytes (FLS) are RANKL and a pro-inflammatory cytokine expressing and producing cells in patients with RA (4). The FLS possess two Fc receptors, FcγRIIa (CD32a) and FcγRI (CD64), which bind to the immunoglobulin and activate FLSs (20, 21). Therefore, ACPA may activate RA-FLS, and regulating ACPA-induced RA-FLS activation may control osteoclastogenesis in patients with RA.

This study aimed to investigate the effects of ACPA on RA-FLS activation. We also used anti-CD32 and CD64 antibodies and PAD-2 and 4 inhibitors to evaluate the regulatory role of these agents in RA-FLS activation. We investigated the influence of ACPA-stimulated RA-FLS on osteoclastogenesis and that of anti-CD32 and CD64 and PAD-2 and 4 inhibitors on ACPA-stimulated RA-FLS-induced osteoclastogenesis.

Methods

Patients

Synovial tissues were obtained from four patients with RA who underwent total knee replacement surgery for medical purposes, and FLSs were isolated from the synovial tissues as described previously (22). Peripheral blood was obtained from four patients with RA aged >18 years who fulfilled the 2010 RA classification criteria (8). This study followed the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all the participants. The experimental protocol was approved by the Institutional Review Board of Konkuk University Medical Center (KUMC 2023-07-024).

In vitro RA-FLS stimulation with ACPA and co-culture with anti-CD32/CD64 and PAD2/4 inhibitors

RA-FLSs (1 × 106 cells/mL) were plat-

ed in 24-well culture plates containing serum-free Dulbecco's modified eagle medium (DMEM) for 24 h. After 24 h of starvation, the culture medium was changed to DMEM supplemented with 10% fetal bovine serum (FBS) and 1, 10, and 100 ng/mL ACPA. The ACPA (monoclonal Ab 12G1, mAb against CCP: HQCHQESTXGRSR-GRCGRSGS; X= citrulline) was manufactured as previously described (23). In addition, 1, 5, and 10 μg/mL anti-CD32 antibody (anti-FcγRIIA antibody, R&D system, AF1875); 1, 5, and 10 μg/mL anti-CD64 antibody (anti-FcγRI antibody, R&D system, MAB1257); 10, 20, and 50 μM PAD-2 inhibitor (CAYMAN chemical company CAY10723); or 100, 200, 500 nM PAD-4 inhibitor (CAYMAN chemical company, CSK199) were added in ACPA 100 ng/mL stimulated condition. As a control non-ACPA monoclonal IgG stimulation, RA-FLSs were stimulated with 100 ng/mL monoclonal recombinant human IgG1 Fc protein (R&D system, 110-HG). RA-FLSs and culture media were harvested 72 h after each media change.

ELISA of the culture media from RA-FLS culture to determine cytokine levels

The levels of interleukin (IL)-17A, IL-6, and IL-1β (R&D Systems, Minneapolis, MN, USA) in the RA-FLSs culture media were measured using enzyme-linked immunosorbent assay following the manufacturer's protocol. The cytokine levels ranged from 10–2,000 pg/mL. A standard curve was drawn by plotting the optical density against the log of the concentration of recombinant cytokines, and this curve was used to determine the cytokine concentrations in test samples.

PAD activity

To assess the inhibitory action of PAD-2 and PAD-4 inhibitors on the PAD activity of RA-FLSs, we used a PAD detection kit (Signalchem biotech, P312-863). The activities of PAD in RA-FLSs were measured in ACPA 0, 1, 10, and 100 ng/mL, ACPA 100 ng/mL with PAD2 inhibitor (10, 20, and 50 μM), and ACPA 100 ng/mL with PAD4 inhibitor (100, 200, and 500 nM) according to the manufacturer's instructions.

Flow cytometry

Harvested RA-FLSs were immunostained using a phycoerythrin (PE)-conjugated anti-RANKL antibody (eBiosciences, San Diego, CA, USA) or PE-conjugated anti-TNF-α antibody (Biolegend) to quantify RANKL+ and tumour necrotic factor-alpha (TNF-α+) in RA-FLSs. The cells were detected using a FACSCalibur flow cytometer (BD Pharmingen, Franklin Lakes, NJ, USA).

Osteoclastogenesis

We obtained peripheral blood mononuclear cells (PBMC) from patients with RA to induce osteoclastogenesis. CD14+ monocytes were extracted from PBMC using the monocyte isolation kit (stemcell). Furthermore, the CD14+ monocytes (8 × 105 cells/ml/well) were primarily seeded onto the 24-well plate in 10% heat-inactivated FBS and 1% penicillin-streptomycin (Gibco) containing α-minimal essential medium (α-MEM; Invitrogen). The suspended CD14+ monocytes were stimulated with 100 ng/mL recombinant macrophage-colony stimulating factor (M-CSF, R&D Systems) for 72 h. After 3 days, the adherent cells were harvested and further cultured with 30 ng/mL M-CSF and soluble receptor activator of NF-κB ligand (RANKL; R&D Systems) in the presence or absence of ACPA (1, 10, and 100 ng/ml). Pre-conditioned osteoclast precursors were cultured with 72 h ACPA (100 ng/ml)-stimulated RA-FLSs (8 × 102 cells/ml/well) with or without various concentrations of anti-CD32a/64 antibody and PAD-2/4 inhibitors to evaluate the influence of ACPA-stimulated RA FLSs and anti-CD32a/64 antibody or PAD-2/4 inhibitors. The culture medium was changed every 72 h. After 10–14 days of culture, the cells were fixed and stained using a tartrate-resistant acid phosphatase (TRAP) staining kit (Kamiya Biomedical Company) following the manufacturer's instructions. TRAP-positive multinucleated cells were counted thrice, and the examiner was blinded to the culture conditions.

RNA preparation and reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted using an easy-spinTM Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Republic of Korea) following the manufacturer's instructions. Furthermore, the RNA samples were quantified, aliquoted, and stored at -80 °C until analysis. Total RNA (500 ng) was reverse transcribed to complementary DNA (cDNA) using an AccuPower CycleScript RT PreMix cDNA synthesis kit (Bioneer, Daejeon, Republic of Korea) following the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qPCR) was conducted using 20 μL of solution containing 7.2 μL of PCR-grade distilled water, 0.4 μL of forward and reverse primers, and 10 μL of the SYBR Green I Master mix (Roche Diagnostics, Mannheim, Germany). PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 59 °C for 15 s, and 72 °C for 15 s. Notably, all primers were synthesised by Bioneer Corp. (Daejeon, Republic of Korea), and the relative messenger RNA (mRNA) expression levels of *TRAP, Cathepsin K, RANK, Nuclear factor-activated T cells, cytoplasmic, calcineurin dependent1 (NFATc1), and adenosine triphosphatase, H+ transporting, lysosomal V0 subunit D2* (*ATP6v0d2*) were normalised to *actin beta* (*ACTB*) mRNA levels.

Statistical analysis

Data were presented as the mean ± standard error of the mean. One-way analysis of variance with *post-hoc* Bonferroni's multiple comparison test was used. Statistical significance was set at $p<0.05$. All statistical analyses were performed using Prism 10.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of ACPA on RA-FLSs and regulation using anti-FcγR antibody or PAD inhibitors

We evaluated the effects of ACPA on RA-FLS activation. The proportion of RANKL+ and TNF-α+ RA-FLSs was increased by the dose-dependent ACPA stimulation and was suppressed by anti-CD64 antibodies (Fig. 1A). The IL-6, IL-1β, and IL-17A levels in the culture media were increased by ACPA stimulation and were reduced by adding

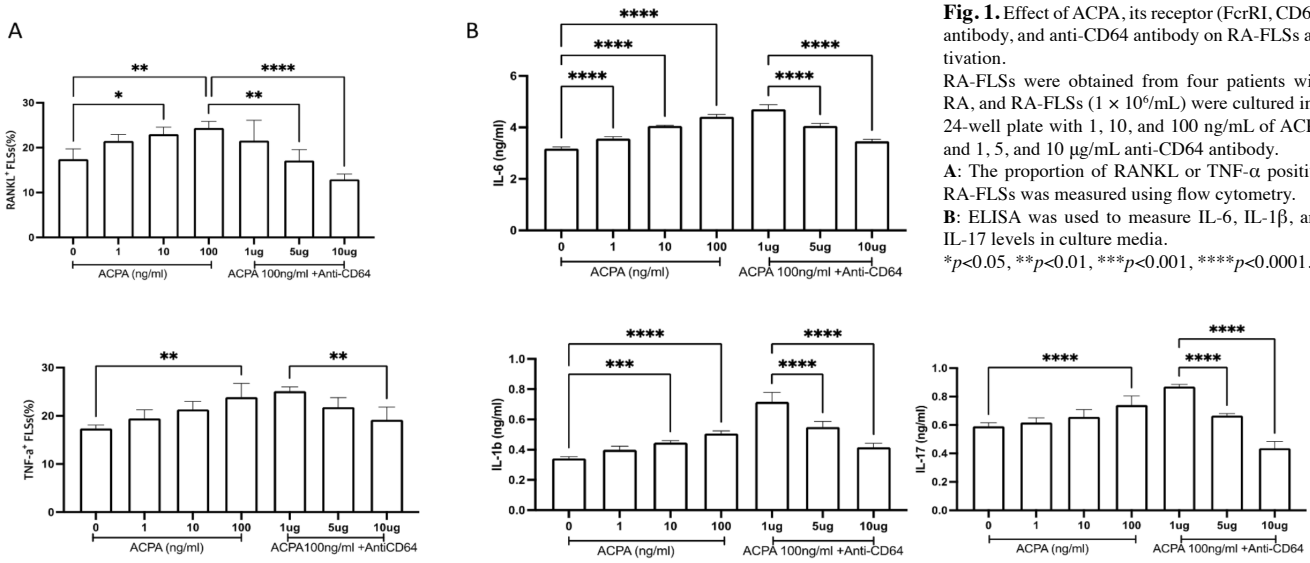

Fig. 1. Effect of ACPA, its receptor (FcrRI, CD64) antibody, and anti-CD64 antibody on RA-FLSs activation.
RA-FLSs were obtained from four patients with RA, and RA-FLSs (1×10^6/mL) were cultured in a 24-well plate with 1, 10, and 100 ng/mL of ACPA and 1, 5, and 10 μg/mL anti-CD64 antibody.
A: The proportion of RANKL or TNF-α positive RA-FLSs was measured using flow cytometry.
B: ELISA was used to measure IL-6, IL-1β, and IL-17 levels in culture media.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

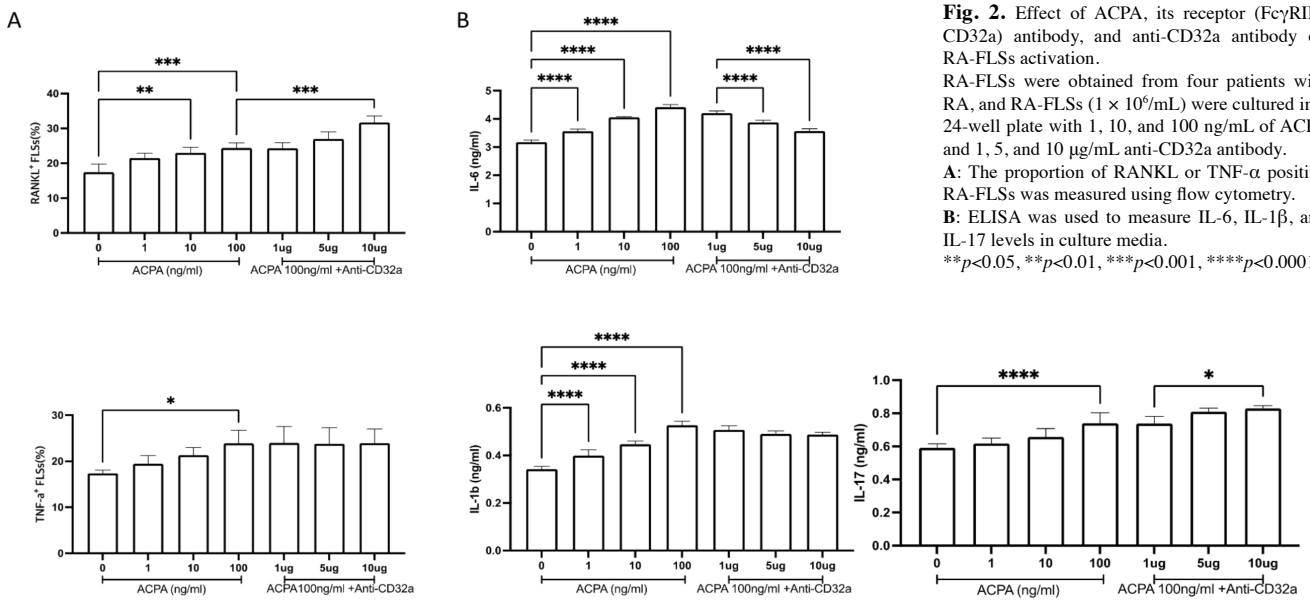

Fig. 2. Effect of ACPA, its receptor (FcγRIIa, CD32a) antibody, and anti-CD32a antibody on RA-FLSs activation.
RA-FLSs were obtained from four patients with RA, and RA-FLSs (1×10^6/mL) were cultured in a 24-well plate with 1, 10, and 100 ng/mL of ACPA and 1, 5, and 10 μg/mL anti-CD32a antibody.
A: The proportion of RANKL or TNF-α positive RA-FLSs was measured using flow cytometry.
B: ELISA was used to measure IL-6, IL-1β, and IL-17 levels in culture media.
**p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

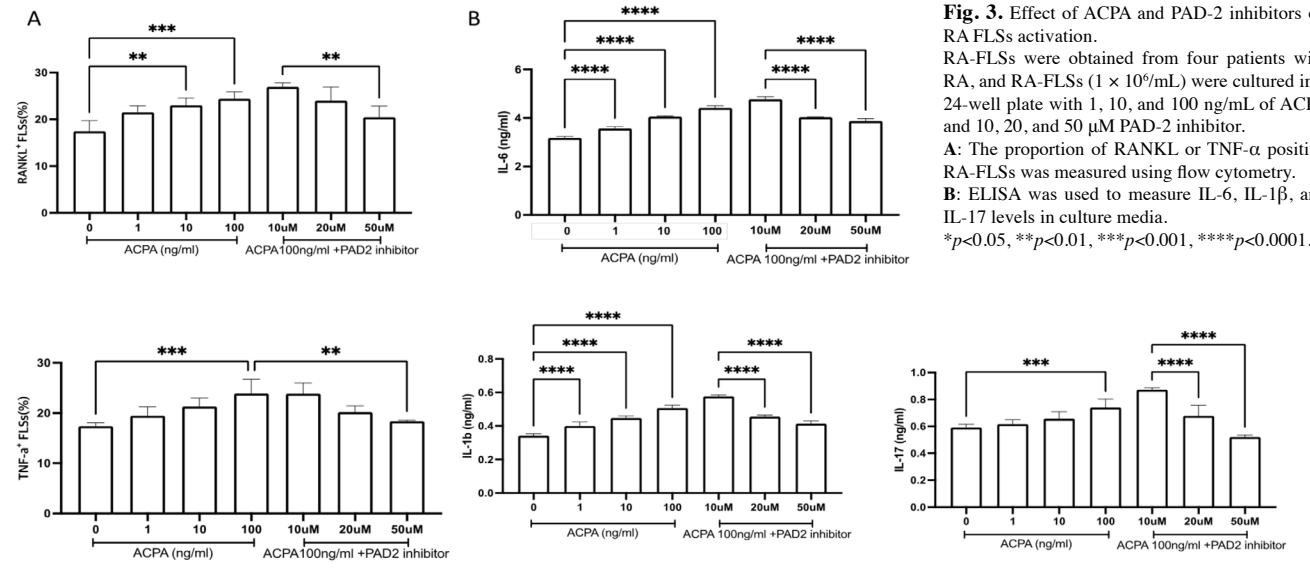

Fig. 3. Effect of ACPA and PAD-2 inhibitors on RA FLSs activation.
RA-FLSs were obtained from four patients with RA, and RA-FLSs (1×10^6/mL) were cultured in a 24-well plate with 1, 10, and 100 ng/mL of ACPA and 10, 20, and 50 μM PAD-2 inhibitor.
A: The proportion of RANKL or TNF-α positive RA-FLSs was measured using flow cytometry.
B: ELISA was used to measure IL-6, IL-1β, and IL-17 levels in culture media.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

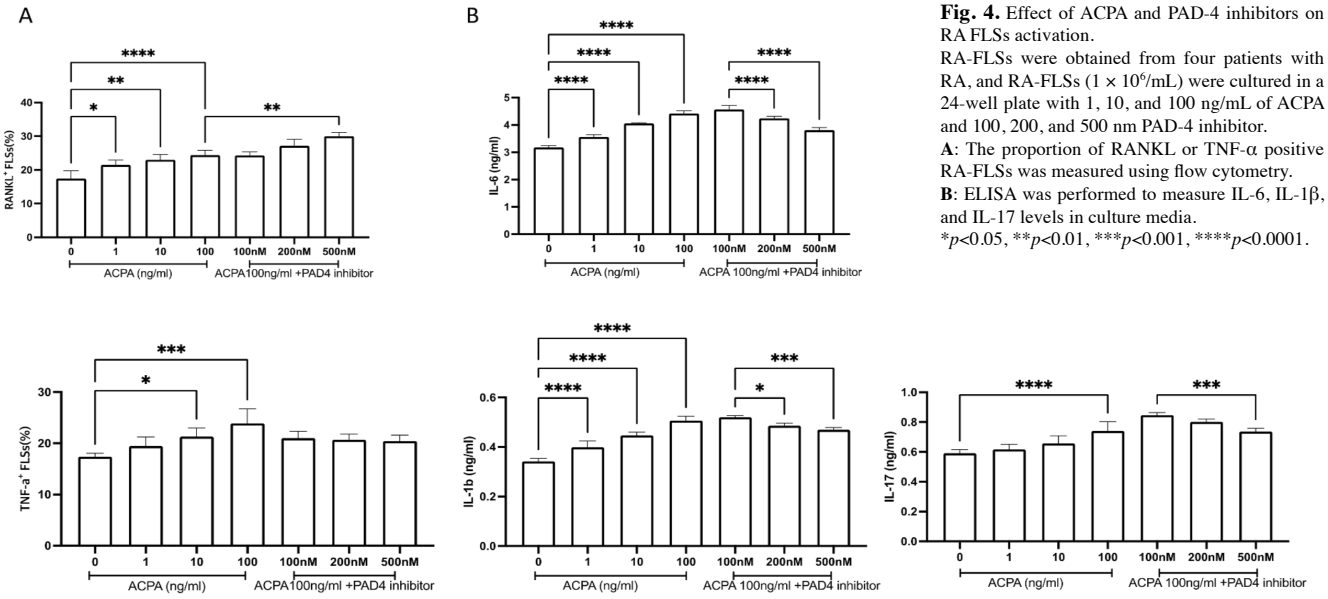

Fig. 4. Effect of ACPA and PAD-4 inhibitors on RA FLSs activation.
RA-FLSs were obtained from four patients with RA, and RA-FLSs (1×10^6/mL) were cultured in a 24-well plate with 1, 10, and 100 ng/mL of ACPA and 100, 200, and 500 nm PAD-4 inhibitor.
A: The proportion of RANKL or TNF-α positive RA-FLSs was measured using flow cytometry.
B: ELISA was performed to measure IL-6, IL-1β, and IL-17 levels in culture media.
*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

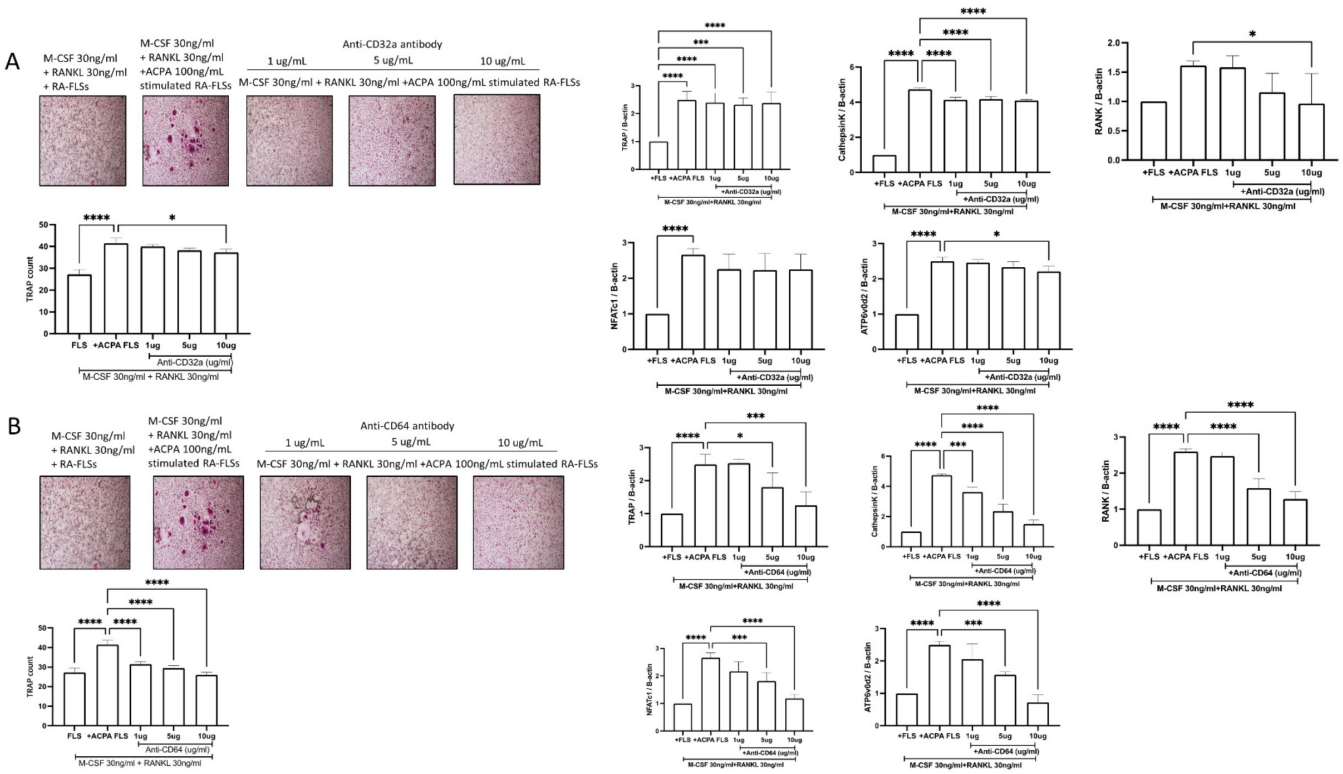

Fig. 5. Effect of anti-FcγR and anti-CD32a/CD64 antibodies on the osteoclast differentiation co-cultured with ACPA pretreated RA-FLSs.
RACD14+ monocytes from four patients with RA (8×10^5/well) were plated in a 24-well plate and stimulated with 100 ng/mL M-CSF for 72 h to induce osteoclast precursor.
A: These were co-cultured with unstimulated RA-FLSs or 100 ng/mL ACPA pre-stimulated RA-FLSs (8×10^2/well). In addition, 1, 5, and 10 μg/mL anti-CD32a antibodies were added to a condition containing ACPA pre-stimulated RA-FLSs.
B: These were also conducted with 1, 5, and 10 μg/mL anti-CD64 antibodies. TRAP-positive multinucleated cells were counted after 10–14 days of culture. Gene expression levels were normalised to expression levels of *β-actin*. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5 and 10 μg/mL anti-CD64 antibodies (Fig. 1B). The proportion of RANKL+ and TNF-α+ RA-FLSs and levels of IL-6, IL-1β, and IL-17A in culture media did not increase with 1, 10, and 100 ng/mL of control monoclonal IgG1 Fc protein stimulation (Supplementary Fig. 1A-B). Adding anti-CD32a antibody did not reduce the proportion of RANKL+ and TNF-α+ RA-FLSs that was initially increased by ACPA stimulation (Fig. 2A). Notably, only the IL-6 levels in the culture media were reduced after adding 5 and 10 μg/mL anti-CD32a antibodies. However, IL-1β and IL-17A levels remained unchanged (Fig. 2B). The proportion of RANKL+ and TNF-α+ RA-FLSs decreased in

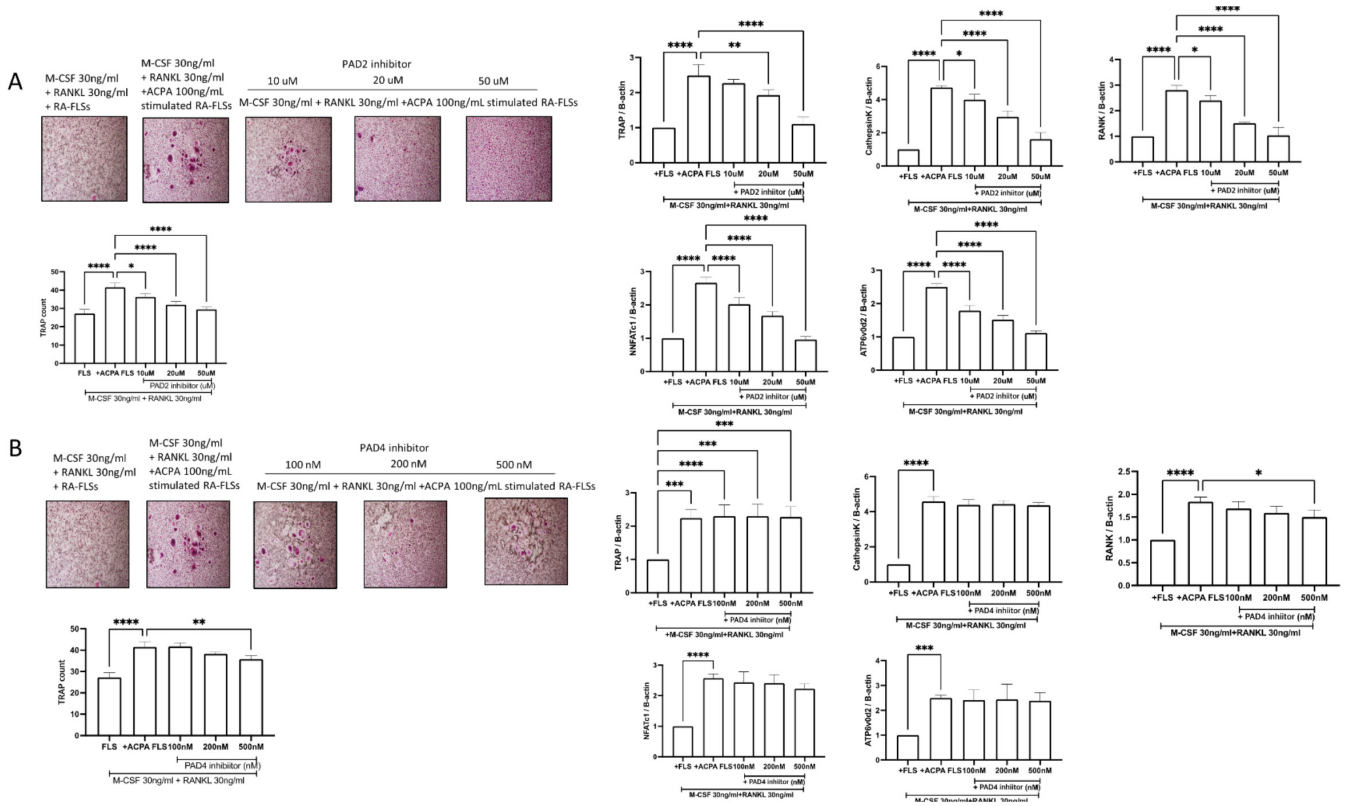

Fig. 6. Effect of PAD-2/4 inhibitors on the osteoclast differentiation co-cultured with ACPA pretreated RA-FLSs.
CD14+ monocytes from four patients with RA (8×10^5/well) were plated in a 24-well plate and stimulated with 100 ng/mL M-CSF for 72 h to induce osteoclast precursor.
A: These were co-cultured with unstimulated RA-FLSs or 100 ng/mL ACPA pre-stimulated RA-FLSs (8×10^2/well). In addition, 10, 20, and 50 μM PAD-2 inhibitors were added to a condition containing ACPA pre-stimulated RA-FLSs.
B: These were also conducted with 100, 200, and 500 nM PAD-4 inhibitors. TRAP-positive multinucleated cells were counted after 10–14 days of culture. Gene expression levels were normalised to expression levels of *β-actin*. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

50 μM condition containing PAD-2 inhibitor (Fig. 3A), whereas PAD-4 inhibitor did not reduce the proportion of RANKL+ and TNF-α+ RA-FLSs (Fig. 4A). Higher doses of PAD-2 and -4 inhibitors decreased the culture media's IL-6, IL-1β, and IL-17A levels (Fig. 3B and 4B). The PAD-2 and PAD-4 activities of RA-FLSs increased in a dose-dependent manner by ACPA stimulation, and each inhibitor of PAD-2 and PAD-4 specifically suppressed their PAD activities (Suppl. Fig. 2A-B).

Effect of ACPA on osteoclastogenesis

We evaluated TRAP+ multinucleated osteoclast counts at various ACPA concentrations to evaluate the direct effect of ACPA on osteoclastogenesis. The culture media conditions were as follows: 1) M-CSF only (negative control); 2) M-CSF + 1, 10, or 100 ng/mL ACPA; and 3) M-CSF + RANKL (positive control). The addition of ACPA resulted in a dose-dependent increase in TRAP+ multinucleated osteoclast counts (Suppl. Fig. 3A), and comparable TRAP+ multinucleated osteoclast counts with positive control (M-CSF + RANKL) were observed with M-CSF + 100 ng/mL ACPA. Furthermore, we evaluated the indirect effect of ACPA on osteoclastogenesis by co-culturing ACPA-pre-stimulated RA-FLSs and osteoclast precursors. These were evaluated using a negative control (M-CSF only), positive control (M-CSF + RANKL stimulation), M-CSF + RANKL + unstimulated RA-FLSs, and M-CSF + RANKL + ACPA pre-stimulated RA-FLSs. TRAP+ multinucleated osteoclast counts were increased in M-CSF + RANKL, M-CSF + RANKL + unstimulated RA FLSs, and M-CSF + RANKL + ACPA pre-stimulated RA FLSs compared with the negative control (Suppl. Fig. 3B). Adding ACPA pre-stimulated RA FLSs, compared with M-CSF + RANKL, further increased the TRAP+ multinucleated osteoclast count (Suppl. Fig. 3B). Reverse transcription-qPCR (RT-qPCR) revealed that adding ACPA pre-stimulated RA-FLSs further increased the expression of osteoclast differentiation-related genes, including cathepsin K, TRAP, RANK, NFATc1, and ATP6v0d2 (Suppl. Fig. 3B). These findings suggested that ACPA exerts direct and indirect effects on osteoclastogenesis.

Effects of anti-FcγR antibody or PAD inhibitors on ACPA-stimulated RA-FLS-induced osteoclastogenesis

Higher doses of the anti-CD32a antibody (10 μg/mL) only mildly reduced TRAP+ multinucleated osteoclast count compared with ACPA pre-stimulated RA-FLSs (Fig. 5A). Adding anti-CD32a, compared with ACPA pre-stimulated RA-FLSs, reduced the TRAP, cathepsin K, RANK, and ATP6v0d2

expression levels (Fig. 6A). The addition of anti-CD64 antibody reduced the number of TRAP+ multinucleated osteoclasts and the expression of osteoclast-related genes (*TRAP, Cathepsin K, RANK, NFATc1, and ATP6v0d2*) in a dose-dependent manner (Fig. 5B).

The PAD-2 inhibitor reduced TRAP+ multinucleated osteoclast counts dose-dependently. Furthermore, osteoclast differentiation-related gene expression was attenuated by the PAD-2 inhibitor (Fig. 6A). TRAP+ multinucleated osteoclast differentiation was mildly suppressed in a condition with higher concentrations of PAD-4 inhibitor, and only RANK expression decreased with a higher dose of the PAD-4 inhibitor (Fig. 6B). Finally, the anti-CD64 antibody and PAD-2 inhibitor showed a more potent suppressive role in ACPA-stimulated RA-FLSs-mediated osteoclastogenesis than anti-CD32a antibody or PAD-4 inhibitors.

Discussion

The present study showed some novel findings: 1) ACPA could directly induce RA-FLSs activation toward inflammatory and osteoclastogenic phenotypes, 2) ACPA-activated RA FLSs augment osteoclastogenesis, and 3) ACPA-stimulated RA-FLSs-mediated osteoclastogenesis may be reduced by blocking the CD64-mediated or PAD-2-induced pathway.

Osteoclasts and FLSs primarily mediate irreversible joint destruction in patients with RA (1, 4). FLSs increase osteoclastogenesis by producing RANKL and several pro-inflammatory cytokines (4). Two FcγRs, CD32 and CD64, were highly expressed in synovium of RA patients, and CD32/64-mediated pathway enhanced RA-FLSs activation and pro-inflammatory cytokine induction by C-reactive protein (CRP) stimulation (21). In CRP-mediated RA-FLS activation, the CD32a-mediated signal was more potent than the CD64-mediated signal (21). In the present study, blocking CD64-mediated signals was more important than blocking CD32a in differentiating RA-FLSs into osteoclastogenic phenotypes. The FcγRI (CD64) and FcγRIIa (CD32a) are known as activating receptors which induce pro-inflammatory cytokine production via immunoreceptor tyrosine-based activation motifs (ITAM) (24). The FcγRIIa (CD32a) pocess intrinsic ITAM motif, but FcγRI (CD64) signalling act by ITAM containing FcRγ (25). FcγRI (CD64) is a high-affinity receptor capable of binding monomeric IgG molecules, whereas, FcγRIIa (CD32a) is a low-affinity receptor that requires high-avidity binding by IgG-immune complexes (20, 24). Although the present study showed that blocking only the FcγRI (CD64) signal was effective in reducing RA-FLSs activation and RA-FLSs induced osteoclastogenesis, the action of FcγRs depends on the type of cells and other co-receptors such as toll-like receptors; therefore, further studies are needed to reveal the detailed action of FcγRs on RA-FLSs. Another study showed that ACPA promoted FLS migration mediated by IL-8, PAD, and phosphoinositide-3-kinase (26). PAD-2 and -4 are the dominantly expressed PAD enzymes in the RA synovium, and the expression levels of PAD-2 and -4 correlate with the inflammation intensity of the RA synovium (7). Most PAD-2 exists in the cytoplasm of cells, but a fraction of them acts inside the nucleus and citrullinates H3 and H4, which implies a potential role in gene regulation (27). PAD-4 is mainly located in the nucleus, and regulate citrullination of various nuclear peptides (6). The most important differences between PAD-2 and PAD-4 are their location, and target peptides. Citrullination of histone H3 arginine 26 (H3R26) is mediated only by PAD-2, but not by PAD-4 (28). Perforin-induced cytotoxic assays in RA patients demonstrated that autoantibody reactivities were different between PAD-2 and PAD-4 generated citrullinated autoantigens (29), indicating that hypercitrullination by each PAD enzyme may have different importance in pathogenesis of RA. We demonstrated that ACPA increased the proportion of RANKL+ and TNF-α+ RA-FLSs and induced the production of proinflammatory cytokines, and these activities were diminished by anti-CD64 antibody and PAD-2 inhibitors. This suggests that ACPA is involved in FLS activation mediated by two different pathways: CD64 (FcγRI)-mediated and PAD2-induced citrullination.

Notably, some studies have demonstrated a direct role of ACPA in osteoclastogenesis. The first study showed that ACPA inhibited citrullinated vimentin-augmented osteoclastogenesis, mediated by TNF-α+ release (19). Krishnamurthy *et al*. showed that ACPA induces osteoclastogenesis through PAD-2-dependent, PAD-4-dependent, and IL-8-mediated pathways (30). ACPA-mediated osteoclastogenesis is not universally induced; only ACPA reactive to citrullinated enolase and vimentin can induce osteoclastogenesis (30). The ACPA used in the present study was reactive against citrullinated collagen, filaggrin, and fibronectin (23), and we demonstrated that ACPA, which is unreactive to vimentin and enolase, could also induce osteoclastogenesis (Fig. 5). In addition, ACPA-stimulated RA-FLSs increased osteoclastogenesis, and this is the first study to demonstrate the indirect FLS-mediated osteoclastogenesis induced by ACPA. Similar to the FLS activation mechanisms of ACPA, ACPA-stimulated RA-FLSs-induced osteoclastogenesis was reduced by anti-CD64 antibody and PAD-2 inhibitors. This indirect role of ACPA on osteoclastogenesis may be explained by osteoclastogenic (RANKL+, TNF-α+) FLSs induction and the release of pro-inflammatory cytokines (IL-1β, IL-6, and IL-17).

The presence of ACPA is a hallmark of the autoimmune-mediated RA mechanism, and its positivity and titre are vital for diagnosing RA (8). In addition, a high ACPA titre is a well-known predictor of poor prognosis in patients with RA (10). Arthralgia can precede the pre-clinical RA stage without definite joint inflammation (1). Wigerblad *et al*. reported that ACPA could promote nociception by inducing CXC motif chemokine ligand 1 in an RA animal model (31). The presence of ACPA precedes clinical RA, and arthralgia in the pre-clinical stage of RA may contribute to ACPA-induced pain signal (31). Depleting autoantibodies is not a treatment target in the current RA guidelines (10); however, the recent pathological roles of ACPA are accumulating, and deplet-

ing ACPA or blocking ACPA-mediated pathways may become future therapeutic goals.

In conclusion, we demonstrated that ACPA can induce osteoclastogenic RA-FLSs, and ACPA-stimulated RA-FLSs can augment osteoclastogenesis. In addition, ACPA-mediated RA-FLS activation and consequent osteoclastogenesis were suppressed by blocking the CD64- and PAD-2-mediated pathways. These findings support a novel pathological role of ACPA in RA pathogenesis. Furthermore, blocking CD64- and PAD-2-mediated pathways may ameliorate ACPA-mediated joint destruction in patients with RA.

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