Differential inflammation-mediated function of prokineticin 2 in the synovial fibroblasts of patients with rheumatoid arthritis compared to osteoarthritis

Kentaro Noda (knoda3353@jikei.ac.jp)
University Hospital Regensburg
Bianca Dufner
University Hospital Regensburg
Haruyasu Ito
Jikei University School of Medicine
Ken Yoshida
Jikei University School of Medicine
Gianfranco Balboni
University of Cagliari
Rainer Straub
University Hospital Regensburg

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Abstract

Prokineticin 2 (PK2) is a secreted protein involved in several pathological and physiological processes, including the regulation of inflammation, sickness behaviors, and the circadian rhythm. Recently, it was reported that PK2 is associated with the pathogenesis of collagen-induced arthritis in mice. However, whether PK2 influences the pathogenesis of rheumatoid arthritis (RA) or osteoarthritis (OA) remains unknown. In this study, we collected synovial tissue, plasma, synovial fluid, and fibroblast-like synoviocytes (FLS) from RA and OA patients to analyze the role of PK2 using immunohistochemistry, ELISAs, and tissue superfusion studies. PK2 and its receptors prokineticin receptor (PKR) 1 and 2 were expressed in RA and OA synovial tissues. PKR1 expression in RA synovial tissue was downregulated compared with OA synovial tissue. The PK2 concentration was higher in RA synovial fluid than in OA synovial fluid but similar between RA and OA plasma. PK2 suppressed the production of IL-6 from TNFα-prestimulated OA-FLS, and this effect was attenuated in TNFα-prestimulated RA-FLS. This phenomenon was accompanied by the upregulation of PKR1 in OA-FLS and the downregulation of PK2 and PKR1 in RA-FLS. This study provides a new model to explain some aspects underlying the chronicity of inflammation in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by the proliferation of synovial cells and extensive inflammatory cell infiltration (pannus). RA patients show immunological abnormalities, including anti-cyclic citrullinated peptide antibody and rheumatoid factor[1]. The main symptoms of RA include tenderness and swelling in multiple joints due to inflammation and joint deformities caused by cartilage and bone destruction. The secondary symptoms are associated with chronic inflammation (depression, appetite loss, insomnia) and impaired health-related quality of life[2].

Factors that regulate neuroendocrine pathways connecting the central nervous system and peripheral tissues, such as hormones, neuropeptides, and sympathetic and sensory nerves, are associated with RA symptoms[3], including morning stiffness[4] and pain[5]. Additionally, these factors often play a role in local inflammation in RA (synovial tissue) as their receptors are expressed in synovial tissue. For instance, increased peripheral metabolism of sex hormones is observed in inflamed synovial tissue[6–8], a thyroid hormone network exists in synovial tissue in patients with osteoarthritis (OA) and RA[9], and substance P from sensory nerve fibers exhibits a proinflammatory role by recruiting leukocytes, promoting their plasma extravasation[10], and stimulating the secretion of proinflammatory cytokines from synovial fibroblasts in synovial tissue[11]. We were interested in elucidating new neuroendocrine pathways and focused on prokineticin 2 (PK2).

PK2 is a secreted protein containing a five-disulfide-bridged motif termed a colipase fold and is involved in the regulation of neuroendocrine pathways[12]. Two types of G-protein-coupled receptors, prokineticin receptor 1 (PKR1) and prokineticin receptor 2 (PKR2)[13], have been identified to interact with PK2, and

both receptors couple to Gαq, Gαs, and Gαi proteins, indicating that PKRs activate multiple signaling pathways[14]. PK2 is expressed in the brain, testis, adrenal gland, uterus, intestine, liver, bone marrow, and blood cells[15,16]. PKR1 is predominantly distributed in peripheral tissue, and PKR2 is mainly distributed in brain tissue[15,17].

PK2 has multiple physiological functions, including the regulation of neurogenesis[18], angiogenesis[19], pain threshold[20,21], mood [22,23], circadian rhythm[24], ingestive behaviors[25–27], and energy expenditure[28] (Fig. 1A). PK2 is also known to act as a proinflammatory factor for inflammatory cells[29,30]. For example, PK2 promotes the migration of mouse macrophages and induces a proinflammatory phenotype through the production of interleukin (IL)-1 and inhibition of IL-10[31], mobilizes neutrophils to the site of inflammation[32], and suppresses the production of IL-10 and IL-4 in mouse splenocytes through the PKR1 pathway[33].

The PK2 concentration in the peripheral blood of patients with inflammatory diseases, such as multiple sclerosis[34] and psoriasis[35], is increased compared with healthy controls. Taking these factors into consideration, the mechanisms underlying the symptoms and pathogenesis of arthritis appear to be similar to the processes regulated by PK2. However, the association between PK2 and the pathogenesis of RA has not yet been elucidated in patients. We previously showed that PK2 expression was upregulated in the joints of mice with collagen-induced arthritis (CIA) [36], and the administration of a PKR antagonist attenuated mouse CIA[37]. Many granulocytes are present in the synovial tissue of mice with CIA[38], whereas most of the influential cells in RA synovial tissue during the chronic phase are synovial cells[39]. Therefore, the effect of PK2 in RA synovial tissue may be different compared with acute inflammatory tissue in mice (Fig. 1B).

Based on the described aspects of PK2, we hypothesized that PK2 is associated with the pathogenesis of RA and that the role of PK2 in RA is different from that in OA.

Materials And Methods

Patients. We conducted a study of 67 patients (19 men, 48 women; mean age = 62.6 ± 10.6 years) with established RA (according to the American College of Rheumatology/European League Against Rheumatism criteria[40]) and 79 patients (31 men, 48 women; mean age 68.0 ± 8.07 years) with OA. All patients underwent total knee arthroplasty in the Department of Orthopedic Surgery, and peripheral blood, synovial fluid, and synovial tissue samples were obtained. The concentration of C-reactive protein in RA and OA patients was 15.8 ± 26.7 mg/l and 2.18 ± 3.02 mg/l, respectively, indicating that the systemic inflammation in OA patients was significantly lower than in RA patients. Among RA patients, 51 received glucocorticoids, 34 methotrexate, 13 biologics, 7 leflunomide, and 3 sulfasalazine. Among OA patients, one patient received glucocorticoids. We performed this study according to the Helsinki Declaration of 1975, as revised in 1983. Approval for this study was obtained from the Ethics Committee of the

University of Regensburg (approval number 15 – 1 01–021). All patients knew the purpose of the study and provided informed consent.

Reagents. Human recombinant PK2 (#100 – 46), tumor necrosis factor α (TNFα) (#300–01A), IL-1β (#200–01B), and transforming growth factor (TGFβ) (#100–21C) were obtained from Peprotech (Rocky Hill, NJ, USA). The PKR1 and PKR2 antagonist[41] [PKRA7 (508942)] was obtained from Merck (Darmstadt, Germany), and the PKR1 antagonist (PC-7)[42] was generated by one of the authors of the current study (G.B.).

Synovial tissue and synovial fibroblast preparation. Synovial tissue samples from patients with RA and OA were obtained immediately after opening the knee joint capsule. Pieces of the synovial tissue of up to 9 cm^2 were excised. One part of the tissue was minced and treated with Liberase TM (#05401127001, Roche Diagnostics, Mannheim, Germany) at 37ºC for 1 h on a shaking platform. The resulting suspension was filtered (70 µm) and centrifuged at 1600 rpm for 10 min. The pellet was then treated with erythrocyte lysis buffer (20.7 g NH_4Cl, 1.97 g NH_4HCO_3, 0.09 g EDTA ad 1 L H_2O) for 8 min and recentrifuged at 1600 rpm for 10 min. The pellet was resuspended in RPMI-1640 (Sigma Aldrich, St. Louis, MO, USA) with 10% fetal calf serum (FCS). After overnight incubation, cells were supplemented with fresh medium. The culture medium used was RPMI-1640 without phenol red (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, 4 mM L-glutamine (Sigma Aldrich, St. Louis, MO, USA), 10 mM HEPES (Sigma Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA), and 10 µg/ml ciprofloxacin (Fresenius Kabi, Bad Homburg, Germany). Passage 4–8 fibroblast-like synoviocytes (FLS) were used for experiments. The other part of the collected tissue was used for a superfusion experiment and immunohistochemistry.

Synovial tissue superfusion. One piece of ~ 16 mm^2 fresh synovial tissue from the patients was loaded into a superfusion chamber (80 µl), as described previously[43]. Then, superfusion was performed for 2 h at 37ºC at a flow rate of 66 µl/min with serum-free culture medium. The superfusate was collected at 2 h and used for ELISA.

Histological analysis. The expression of PK2, PKR1, and PKR2 in synovial tissues and FLS from patients with OA and RA was analyzed by immunohistochemistry. For synovial tissue staining, the tissues were fixed in 3.7% formalin and embedded in paraffin. The paraffin block was sectioned into 6–8 µm slices and used for staining. The sections were incubated in Dako Target Retrieval Solution (S1699, Agilent, Santa Clara, CA, USA) at 90ºC for 1 h to activate the antigens. Endogenous peroxidase was inactivated with 3% H_2O_2 in methanol. The sections were then blocked with 10% normal goat serum, 10% bovine serum albumin (BSA), and 10% FCS for 1 h and incubated with primary antibodies (rabbit anti-PK2 polyclonal antibody [bs-5784R, 2.5 µg/ml] from Bioss antibodies Woburn, MA, USA; rabbit anti-PKR1 [NBP1-83337, 2 µg/ml] and anti-PKR2 polyclonal antibody [NBP1-92290, 1 µg/ml] from Novus Biologicals, via Bio-Techne, Wiesbaden, Germany) overnight at 4ºC. Subsequently, the sections were incubated with polyclonal goat anti-rabbit immunoglobulins/HRP (P0448, 0.5 µg/ml) from Agilent, Santa Clara, CA, USA, for 1 h at room temperature. The color was then developed by incubation with ImmPACT

DAB (SK-4105) from Vector laboratories, CA, USA, for 10 min at room temperature. The sections were finally counterstained with hematoxylin.

FLS from OA and RA patients were cytospined and fixed with cold acetone. The cytospin sections were blocked with 10% normal goat serum and 10% BSA and then incubated with primary antibodies (rabbit anti-PK2 polyclonal antibody [ab76747, 5 µg/ml] from Abcam, Cambridge UK; rabbit anti-PKR1 polyclonal antibody [NBP1-83337, 1 µg/ml] from Novus Biologicals, via Bio-Techne, Wiesbaden, Germany; and mouse anti-PKR2 monoclonal antibody [sc-365696, 4 µg/ml] from Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4ºC. Subsequently, cytospins were incubated with an Alexa Fluor 488-labeled secondary antibody (A-11070, 2 µg/ml) from Thermo Fisher, Schwerte, Germany. The sections were finally counterstained with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI). Control experiments were performed with isotype antibodies instead of the primary antibody.

The sections were examined under a microscope (BX-61, Olympus, Tokyo, Japan). A semiquantitative scoring system was used to analyze the expression of PK2, PKR1, and PKR2 in the synovial tissue. PK2-, PKR1-, and PKR2-positive cells in the lining and sublining layer were counted in 5 fields per sample under a high-power field. The percent positivity was calculated and graded on 0–4 scale: 0 = no stained cells, 1 = 0–25% stained cells, 2 = 26–50% stained cells, 3 = 51–75% stained cells, and 4 = 76–100% stained cells[44].

Cell-based ELISA. In order to study the cellular expression of PK2, PKR1, and PKR2 under proinflammatory conditions, 1×10^4 cells per well were seeded in a 96-well plate and stimulated with TNFα (10 ng/ml), IL-1β (200 pg/ml), and TGFβ (10 ng/ml) for 24 and 48 h. Then, cells were fixed with 3.7% formalin (for PK2) for 20 min or cold methanol for 10 min (for PKR1 and PKR2). Formalin-treated cells were permeabilized and blocked with 0.1% Triton-X and 1% BSA in PBS for 1 h. Methanol-treated cells were blocked with 1% BSA in PBS for 1 h. After blocking, cells were incubated with primary antibodies overnight at 4ºC. The antibodies and concentrations used were the same as those described above for immunohistochemistry in FLS. Cells were incubated with a polyclonal HRP-conjugated goat anti-rabbit secondary antibody (#32260, 0.5 µg/ml for PKR1 and PKR2, 0.25 µg/ml for PK2) from Thermo Fisher, Schwerte, Germany for 1 h at room temperature and visualized with 1-step Ultra TMB (#34029 from Thermo Fisher, Schwerte, Germany). After stopping the reaction with 2 M sulfuric acid, the optical density was determined using a Biorad imark™ microplate reader (Bio-rad, München, Germany).

In order to study the phosphorylation of nuclear factor kappa B (NFκB) p65 after stimulation with PK2 under proinflammatory conditions, 1×10^4 cells per well were seeded in a 96-well plate, prestimulated with PK2 (10^{-11} M) for 1 h, and then stimulated with TNFα (10 ng/ml) for 0, 5, 15, 30, 60, and 120 min. Cells were fixed with 3.7% formalin for 20 min and then permeabilized and blocked with 0.3% Triton-X and 1% BSA in PBS for 1 h. After blocking, cells were incubated with an anti-phospho-NFκB p65 (Ser536) rabbit monoclonal antibody (#3033, 1:200) from Cell Signaling Technology, Danvers, MA, USA overnight at 4ºC. Cells were incubated with a polyclonal HRP-conjugated goat anti-rabbit secondary antibody (2.5 µg/ml) from Thermo Fisher for 1 h at room temperature and visualized with 1-step Ultra TMB from

Thermo Fisher. After stopping the reaction with 2 M sulfuric acid, the optical density was determined using a Bio-rad imark™ microplate reader (Bio-rad).

Stimulation of FLS. To study the effect of PK2 on FLS under proinflammatory conditions, 1×10^4 cells per well were seeded in a 96-well plate, prestimulated with TNFα (10 ng/ml) in RPMI-1640 medium containing 2% FCS for 48 h, and then stimulated with medium containing the respective compounds (PC-7 at 1 µM or PKRA7 at 2 µM) and PK2 at concentrations from 10^{-11} M to 10^{-14} M. After 24 h, supernatants were collected and used for ELISAs.

ELISAs. IL-6, matrix metalloproteinase-3 (MMP-3), osteoprotegerin (OPG), and tissue inhibitor of metalloprotease-1 (TIMP-1) levels in cell supernatants were measured using a standard quantitative sandwich ELISA following the manufacturer's protocol (for IL-6, BD OptEIA, BD Biosciences, Heidelberg, Germany; for MMP-3, OPG ,and TIMP-1, DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). PK2 levels in the plasma, synovial fluid, and superfusate from patients with OA and RA were measured using an ELISA kit from Cloud-Clone Corp., Katy, TX, USA, following the manufacturer's protocol.

MTT assays, migration assays, chemotactic assays, and animal experiments. These methods are described in the Supplementary Methods.

Statistical analysis. All data are presented as the mean ± SD. Box plots demonstrate the 10th, 25th, median, 75th, and 90th percentiles. The Mann–Whitney U test was used for two-group comparisons, and the Wilcoxon signed-rank test was used for pairwise comparisons. A one-sample Wilcoxon signed-rank test (when normality was not given) or one-sample t-test (when data were normally distributed) was used to compare the expression of PK2, PKR1, and PKR2 (% of control) in cell-based ELISAs or the concentration of IL-6, MMP-3, TIMP-1, and OPG (% of control) in ELISAs with a fixed population control level of 100%. Spearman rank correlation was used to analyze the correlation between plasma and synovial fluid levels in OA and RA patients. For comparisons between the DMSO control and PC-7 or PKRA7 group in ELISAs, a two-way ANOVA followed by the Bonferroni post hoc test was used. These analyses were conducted with SigmaPlot V.13 (Systat Software, Erkrath, Germany) and GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Results

PK2, PKR1, and PKR2 expression in OA and RA synovial tissue. In previous studies, we showed the presence of PK2, PKR1, and PKR2 proteins in the synovial tissue of mice with CIA[36,37]. Therefore, we first examined PK2, PKR1, and PKR2 expression in OA and RA synovial tissue (Fig. 2). A proportion of mononuclear cells showed positive PK2 expression in these tissues (Fig. 2A), and there was no significant difference in the PK2 positivity rate in the lining and sublining layers between OA and RA tissues (Fig. 2B). Most mononuclear cells in OA tissues and a proportion of mononuclear cells in RA tissues were positive for PKR1, and the PKR1 positivity rates in the OA lining and sublining layers were significantly higher than those in RA. In addition, most mononuclear cells in OA and RA synovial tissues

exhibited positive PKR2 expression, and there was no significant difference in the positivity rate in the lining and sublining layers between OA and RA tissues. Collectively, our data demonstrate that PK2, PKR1, and PKR2 were expressed in RA and OA tissues. In addition, PKR1 expression in RA synovial tissue was downregulated compared with that in OA synovial tissue.

PK2, PKR1, and PKR2 expression in OA- and RA-FLS. Next, we examined PK2, PKR1, and PKR2 expression levels in FLS collected from OA and RA synovial tissues (Fig. 3). Positive expression of PK2 and PKR1 was observed in OA- and RA-FLS (Fig. 3, left and middle panels), whereas minimal PKR2 expression was detected in OA- and RA-FLS (Fig. 3, right panel).

Modulation of PK2, PKR1, and PKR2 expression in OA- and RA-FLS under proinflammatory conditions.

Generally, synovial tissue in RA patients is continuously exposed to a variety of proinflammatory cytokines, whereas synovial tissue in OA patients is less exposed to proinflammatory cytokines. Therefore, we examined the change in PK2, PKR1, and PKR2 expression following stimulation with TNFα, IL-1β, and TGFβ in OA- and RA-FLS using cell-based ELISAs (Fig. 4). PK2 expression in OA-FLS was not influenced by IL-1β, TNFα, or TGFβ at 24 and 48 h after stimulation. Meanwhile, PK2 expression in RA-FLS was downregulated at 24 h after stimulation with IL-1β, TNFα, and TGFβ, and this downregulation lasted for 48 h after stimulation with IL-1β only (Fig. 4A). IL-1β, TNFα, and TGFβ had no effect on PKR1 expression in OA- and RA-FLS at 24 h after stimulation. However, PKR1 expression was upregulated at 48 h after stimulation with TNFα and TGFβ in OA-FLS and downregulated at 48 h after stimulation with IL-1β in RA-FLS (Fig. 4B). PKR2 expression in OA- and RA-FLS was upregulated at 24 and 48 h after stimulation with TGFβ and at 48 h after stimulation with TNFα in RA-FLS (Fig. 4C). Similar to the result of cell-based ELISAs, positive PKR2 expression was detected by immunohistochemistry in OA- and RA-FLS after stimulation with TGFβ (Fig. 4D). These data indicate that the expression of PKR1 under proinflammatory conditions was inversely regulated in OA- and RA-FLS, and these findings correspond to the immunohistochemistry results in OA and RA synovial tissues.

PK2 concentration in plasma, synovial fluid, and superfusate in patients with OA and RA. The mean plasma PK2 concentration in patients with OA and RA was $1.07 \pm 1.47 \times 10^{-9}$ M and $0.97 \pm 0.92 \times 10^{-9}$ M, respectively, and was not statistically different between the patient groups (Fig. 5A, left panel). The mean synovial fluid PK2 concentration in patients with OA and RA was $8.36 \pm 1.31 \times 10^{-11}$ M and $3.50 \pm 7.37 \times 10^{-10}$ M, respectively, and was significantly higher in RA patients than in OA patients (Fig. 5A, right panel).

The superfusate PK2 concentration was below the detection limit of the ELISA Kit. In a direct pairwise comparison, the PK2 concentration was substantially lower in synovial fluid than in plasma in patients with OA and RA (Fig. 5B). Moreover, the PK2 concentration in plasma was significantly correlated with that in synovial fluid in OA patients but not in RA patients (Fig. 5C). These results indicate that PK2 in the synovial fluid was mainly from plasma (blood exudate) in OA patients, whereas PK2 in the synovial fluid in RA patients was produced locally in the synovial cavity in addition to blood exudate.

PK2 has an anti-inflammatory effect in OA-FLS but not RA-FLS. Based on the results of PK2 ELISAs in plasma, synovial fluid, and superfusate, we predicted that PK2 would be present at a low concentration of less than 10^{-11} M in synovial tissue. Because the expression levels of PK2 and PKR1 were modified under proinflammatory conditions, we next investigated the effect of PK2 on OA- and RA-FLS under proinflammatory conditions. We stimulated TNFα-pretreated OA- and RA-FLS with 10^{11} to 10^{-14} M PK2 and measured the supernatant concentration of the arthritis-aggravating factors IL-6 and MMP-3 and arthritis-inhibiting factors TIMP-1 and OPG (Fig. 6). PK2 strongly suppressed IL-6 secretion from OA-FLS in a concentration-dependent manner, and the effect was antagonized by the PKR1-preferential antagonist PC-7 (Fig. 6A). PK2 mildly suppressed IL-6 secretion from RA-FLS; however, the effect was not antagonized by PC-7. MMP-3 secretion from OA- and RA-FLS was suppressed by PK2 in a concentration-dependent manner, and the effect was antagonized by PC-7 (Fig. 6B). TIMP-1 and OPG secretion from OA- and RA-FLS were suppressed by PK2 in a concentration-dependent manner; however, the effect was not antagonized by PC-7 (Fig. 6C and 6D).

We also assessed the antagonizing effect using PKRA7, which is a PKR1 and PKR2 antagonist. The results were similar to those obtained with PC-7, except the inhibitory effect of MMP-3 was not antagonized by PKRA7 in RA-FLS (Supplemental Fig. 1). These data indicate that PK2 inhibits proinflammatory but not anti-inflammatory pathways, especially in OA-FLS. PKR2 expression in OA-FLS was particularly low after stimulation with TNFα, as shown in Figs. 3 and 4C. In addition, the effects of PC-7 and PKRA7 were similar. Therefore, the inhibitory effect on IL-6 and MMP-3 might be exerted through the PKR1 pathway but not the PKR2 pathway.

PK2 does not affect the migration or proliferation of OA- and RA-FLS. It has previously been reported that PK2 affects the migration and proliferation of various cells, including astrocytes[45] and macrophages[31]. We examined cell migration using a scratch assay and cell viability using the MTT assay in OA- and RA-FLS (Supplemental Fig. 2). PK2 at 10^{-11} M did not affect the cell migration or cell viability of OA- and RA-FLS (Supplemental Fig. 2A and 2B).

The anti-inflammatory effect of PK2 in OA-FLS was mediated by NFκB signaling. The production of IL-6 and MMP-3 was attenuated in TNFα–prestimulated OA-FLS by PK2, as shown in Fig. 6 and Supplemental Fig. 1. Regarding the mechanism underlying the anti-inflammatory effect of PK2 in OA-FLS, we hypothesized that PK2 inhibits signaling pathways downstream of TNFα[46]. To test this hypothesis, we examined the expression of phospho-NFκB p65 induced by TNFα in control or PK2 prestimulated OA- or RA-FLS using cell-based ELISAs (Fig. 7). In control prestimulated OA-FLS, phospho-NFκB p65 expression was upregulated at 5, 30, 60, and 120 min after TNFα stimulation (Fig. 7A). In contrast, in PK2 prestimulated OA-FLS, phospho-NFκB p65 expression was not upregulated at any of the time points after TNFα stimulation and was downregulated at 30 min after stimulation (Fig. 7B).

In both control and PK2 prestimulated RA-FLS, phospho-NFκB p65 expression was upregulated at 120 min after TNFα stimulation (Fig. 7C and 7D). Taken together, 30 min after TNFα stimulation, PK2

pretreatment significantly attenuated phospho-NFκB p65 expression in OA-FLS but not RA-FLS compared with the control pretreatment (Fig. 7E).

PK2 mobilized granulocytes into joint spaces and promoted inflammation. As shown in Fig. 5A, the synovial fluid PK2 concentration in RA was higher than in OA. PK2 showed a chemotactic effect in polymorphonuclear (PMN) cells but not monocytes *in vitro* (Supplemental Fig. 3A). Generally, the number of PMN cells in RA synovial fluid is increased compared with OA synovial fluid[47], and many reports have shown that PMN cells produce PK2[16,19,21,48]. We hypothesized that PK2 expressed in PMN cells mobilizes these cells into the synovial fluid. To test this hypothesis, we injected PK2 into healthy mouse knee joints and examined the degree of inflammation and cell infiltration.

After injecting PK2 in the knee joint, we observed an increase in the knee circumference and extensive inflammatory cell infiltration into tissues (Supplemental Fig. 3B). Most of the localized cells were Gr-1/Ly6G-positive, which is a granulocyte marker (Supplemental Fig. 3C), and F4/80-negative, which is a macrophage marker (Supplemental Fig. 3D). These findings correspond to the results of *in vitro* chemotaxis assays (Supplemental Fig. 3A). Taken together, our data indicated that PK2 mobilized PMN cells into joint tissues and caused inflammation.

Discussion

For the first time, this study demonstrated a possible role of PK2 in OA and RA. The effect of PK2 was different depending on the cell type (FLS and PMN cells). PK2 had a chemotactic and proinflammatory effect on PMN cells, which has also been reported in previous reports[21,29,32]. Therefore, we predicted that PK2 would also have a proinflammatory effect on FLS. However, PK2 exhibited an anti-inflammatory effect on TNFα-prestimulated FLS, and NFkB appeared to play a role. Moreover, the effect was diminished in RA-FLS compared with OA-FLS, and the responsiveness of PK2 to cytokines in OA-FLS and unresponsiveness of PK2 in RA-FLS were accompanied by an upregulation of PK2 and PKR1 in OA and a downregulation of PKR1 in RA, respectively. This result was consistent with the downregulation of PKR1 in RA synovial tissue compared with OA synovial tissue in immunohistochemistry assays.

This inverse regulation of PKR1 between RA-FLS and OA-FLS likely contributed to the varying responsiveness of PK2 in RA- versus OA-FLS exposed to inflammatory cytokines. The results prompted us to generate a model (Fig. 8). When OA synovial tissue is exposed to inflammatory cytokines in specific clinical situations, including systemic inflammation, injury, and intense exercise, inflammation caused by the exposure often resolves spontaneously. In this situation, PK2 might be a critical factor responsible for the inhibition of inflammation (Fig. 8A).

In contrast, RA synovial tissue is continuously exposed to inflammatory cytokines, leading to the downregulation of PK2 and PKR1 in RA-FLS. As a result, the endogenous inflammation-mediated modulation of PK2 is impaired, and inflammation persists in RA synovial tissue (Fig. 8B). Taken together,

this dysregulation in the endogenous inflammation-mediated modulation of PK2 and its receptor PKR1 in RA-FLS may partially explain the chronicity of inflammation in the pathogenesis of RA.

To understand how the inflammation-mediated modulation system of PK2 functions in OA-FLS, we first determined which of the two receptors are associated with the anti-inflammatory effect of PK2 in OA-FLS. Our results showed that PKR1 but not PKR2 was expressed in unstimulated OA-FLS, and no change in the PKR2 expression level was observed when OA-FLS were stimulated with TNFα or IL-1β. Therefore, PKR2 expression was thought to be substantially lower than PKR1 expression. In addition, the effects of PC-7, which is a PKR1-preferential antagonist, and PKRA7, which is a PKR1 and PKR2 antagonist, were similar. For these reasons, the anti-inflammatory effect of PK2 on OA-FLS is probably mediated through the PKR1 pathway but not the PKR2 pathway.

Second, we investigated how PKR1 downstream signaling influences the anti-inflammatory pathway. We demonstrated that PK2 inhibited NFκB signaling, one of the proinflammatory downstream signaling pathways in OA-FLS. However, the mechanism by which PKR1 downstream pathways inhibit NFκB signaling remains unknown. PKR1 couples to Gαq, Gαs, and Gαi proteins. Many reports of anti-inflammatory effects via G protein-coupled receptors have been reported. For instance, Gαq-coupled receptors activate AMPK[49]. The activation of AMPK suppresses the inflammatory response in synovial fibroblasts[50,51] and rapidly inhibits TNFα-stimulated IKK/IκB/NFκB signaling in adipocytes[52]. Regarding Gαs-coupled receptors, an increase in cAMP suppresses the proinflammatory response via the PKA/CREB pathway in different cell types[52 – 55]. Therefore, we speculated that one or multiple G-protein pathways might be associated with the inhibition of NFκB phosphorylation. However, there is no report on the anti-inflammatory effect of PKR1 downstream signaling pathways to date. Thus, further investigations to address these issues are warranted.

Determining how PK2 contributes to the development of arthritis is challenging for two reasons. First, the expression of PKRs varies depending on the microenvironment and cell type[15,56]. In the present study, we demonstrated the presence of PKR1 but not PKR2 in FLS from OA and RA patients. Indeed, in human synovial tissue, not only PK2- and PKR1-positive cells but also PKR2-positive cells were identified. The presence of PKR2-positive cells shows that PK2 acts on both PKR1 and PKR2 in human synovial tissue because the affinities of PKR1 and PKR2 are similar[13]. Furthermore, the expression of both receptors can be altered by different microenvironmental conditions, including exposure to inflammatory cytokines, as we showed in this study. However, the role of PKR2-expressing cells is not clear.

Previously, we demonstrated that PKR1 and PKR2 were expressed in mice with CIA, and the severity of arthritis was correlated with the expression level of PKR2 rather than PKR1[37]. Moreover, PKRA7 (a PKR1 and PKR2 antagonist; IC□□ = 5 nM and 8.2 nM for PKR1 and PKR2, respectively) suppressed the severity of arthritis in the same model[37]. This was in contrast to the anti-inflammatory effect of PK2 on FLS via PKR1 in the present study. Given these points, PKR2 might have a stronger proinflammatory effect compared with the anti-inflammatory effect of PKR1 in the development of arthritis.

We showed that PKR2 expression was clearly induced by TGFb in OA- and RA-FLS. TGFb has a pro- or anti-inflammatory effect depending on the microenvironment. For instance, TGFb promotes the production of extracellular matrix proteins, such as type II collagen and aggrecan, in chondrocytes and has protective effects on cartilage, whereas it promotes cartilage degradation when expressed with proinflammatory cytokines, including IL-1 and TNFa[57]. In addition, TGFb induces regulatory T cell differentiation, which has an anti-inflammatory effect, whereas it induces Th17 cell differentiation when present with IL-6, which has a proinflammatory effect[58]. Thus, the microenvironment dictates the pro- or anti-inflammatory activity of a cytokine. The effect of PKR2 induced by TGFb also might be modified according to the local environmental factors. Further exploration of the role of PKR2-expressing cells in synovial tissue is needed to elucidate the role of PK2 in arthritis.

Second, the cell types contributing to arthritis vary during different phases of the disease. Generally, granulocytes are increased in the synovial fluid of patients with active early arthritis[47] and are also the main source of PK2[16,19,21,48]. Our results showed that the injection of PK2 into the joints of mice induced the migration of granulocytes and promoted inflammation at the injection sites (Supplemental Fig. 3), and the concentration of PK2 in RA synovial fluid was higher than that in OA synovial fluid. Considering these facts, we speculate that the granulocytes in RA synovial fluid produce PK2 and that PK2 secreted from granulocytes mobilize these cells into the synovial fluid and induce inflammation. This phenomenon could occur in the synovial tissue and synovial fluid of acute arthritis models, including the early phase of RA[59], crystal-induced arthritis[60], infectious arthritis[60], an early phase of mouse CIA[38], and collagen antibody-induced arthritis[38], as most of the influential cells in these arthritides are granulocytes.

The anti-inflammatory effect of PKRA7 in mouse CIA in the previous report[37] might be due to the suppressed migration of granulocytes from the bone marrow during the early phase of arthritis. Meanwhile, infiltrating granulocytes are reduced in the synovial tissue of chronic arthritis models compared with acute arthritis[61]. As a result, the main source of PK2 in synovial tissue changes from granulocytes to synovial cells. It has been suggested that PK2 acts locally on synovial cells at a very low concentration in an autocrine or paracrine manner based on the results of superfusion assays and reduces the secretion of IL-6 from FLS. This may occur in chronic arthritis, including the chronic phase of RA, OA, and mouse CIA. Therefore, PK2 has an ambivalent effect in arthritis that is dependent on the effector cell type, the phase of disease development, and the microenvironment in the presence or absence of additional cytokine stimulators.

In conclusion, we demonstrated that PK2, PKR1, and PKR2 were expressed in synovial tissue. This indicates that PK2 acts locally. Indeed, PK2 had an anti-inflammatory effect on OA-FLS that was likely mediated through the PKR1 pathway, whereas this anti-inflammatory effect was attenuated in RA-FLS because of PKR1 downregulation. This study provides a new model to explain some aspects regarding the chronicity of inflammation in RA. However, the effect of PK2 in the synovial tissue may vary depending on the effector cell type or receptor expression. For this reason, explaining the effect of PK2 on arthritis remains challenging.

Declarations

Acknowledgments

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Author contributions

KN and RHS made substantial contributions to study conception and design. KN, BD, HI, and KY made substantial contributions to data acquisition. KN and RHS made substantial contributions to the analysis and interpretation of data. KN and RHS contributed to drafting the article or critically revising it. GB provided materials for this research. KN, BD, GB, HI, KY, and RHS approved the final version to be published.

Additional Information

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and analyzed for the present study are available from the corresponding author on reasonable request.

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Figures

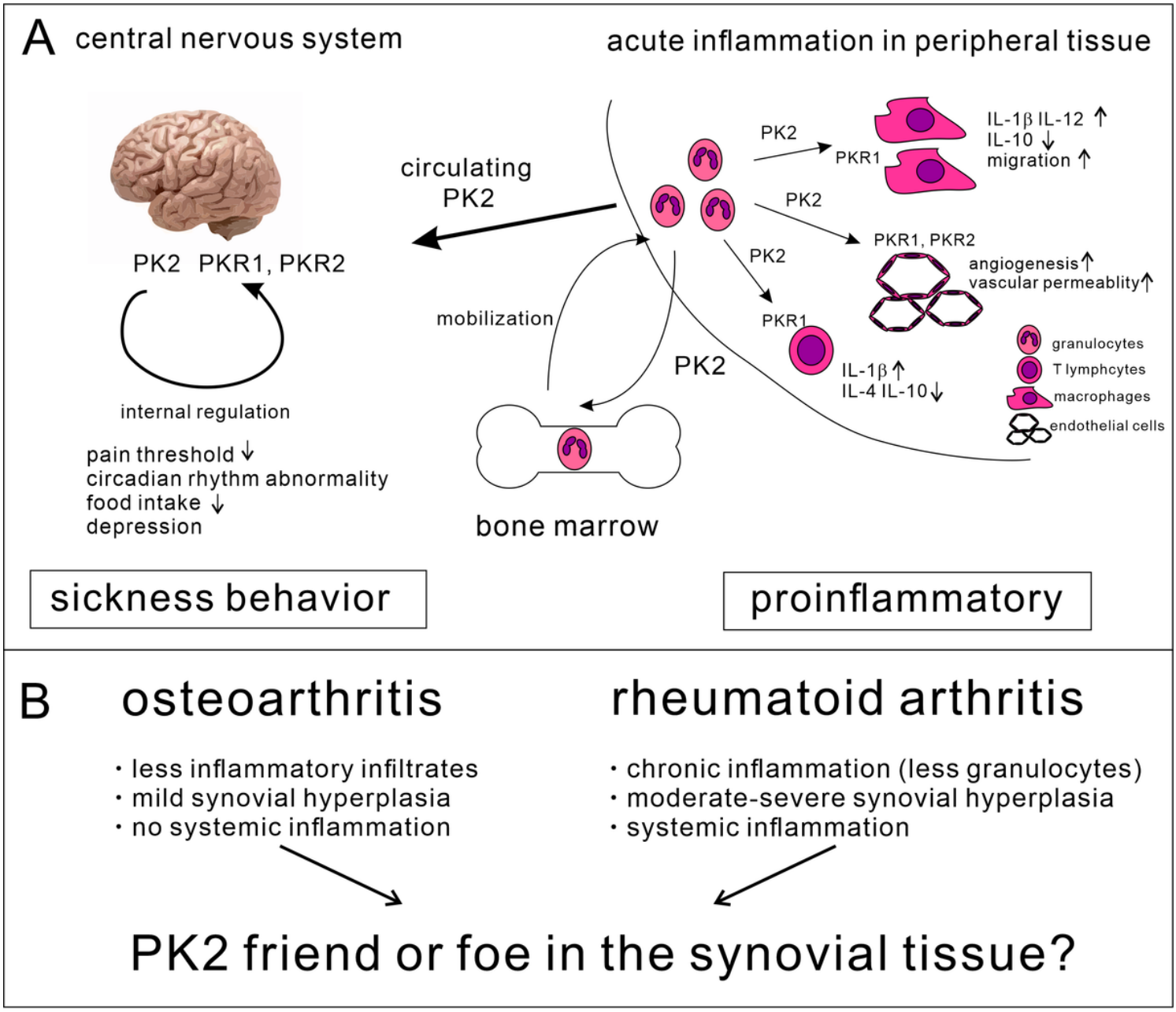

Figure 1. Prokineticin 2 as a regulator of inflammation and sickness behaviors.

Figure 1

Prokineticin 2 as a regulator of inflammation and sickness behaviors. (A) In acute inflammatory tissue, PK2 is secreted by infiltrating granulocytes. Secreted PK2 mobilizes granulocytes from the bone marrow, promotes the production of proinflammatory cytokines from macrophages and T cells through PKR1, and increases angiogenesis and vascular permeability through PKR1 and PKR2, indicating that PK2 acts as a proinflammatory factor. Secreted PK2 reaches the central nervous system through the bloodstream. It regulates the pain threshold, circadian rhythm, food intake, and mood status through PKR1 and PKR2, indicating that PK2 acts as a promotive factor of sickness behaviors under inflammatory conditions. However, in the synovial tissue of rheumatoid arthritis, the most influential cells are synovial cells, not granulocytes (B). Therefore, the effect of PK2 in rheumatoid arthritis and osteoarthritis may be different

from that in acute inflammatory tissue. Abbreviations: PK2, prokineticin 2; PKR1, prokineticin receptor 1; PKR2, prokineticin receptor 2; IL, interleukin.

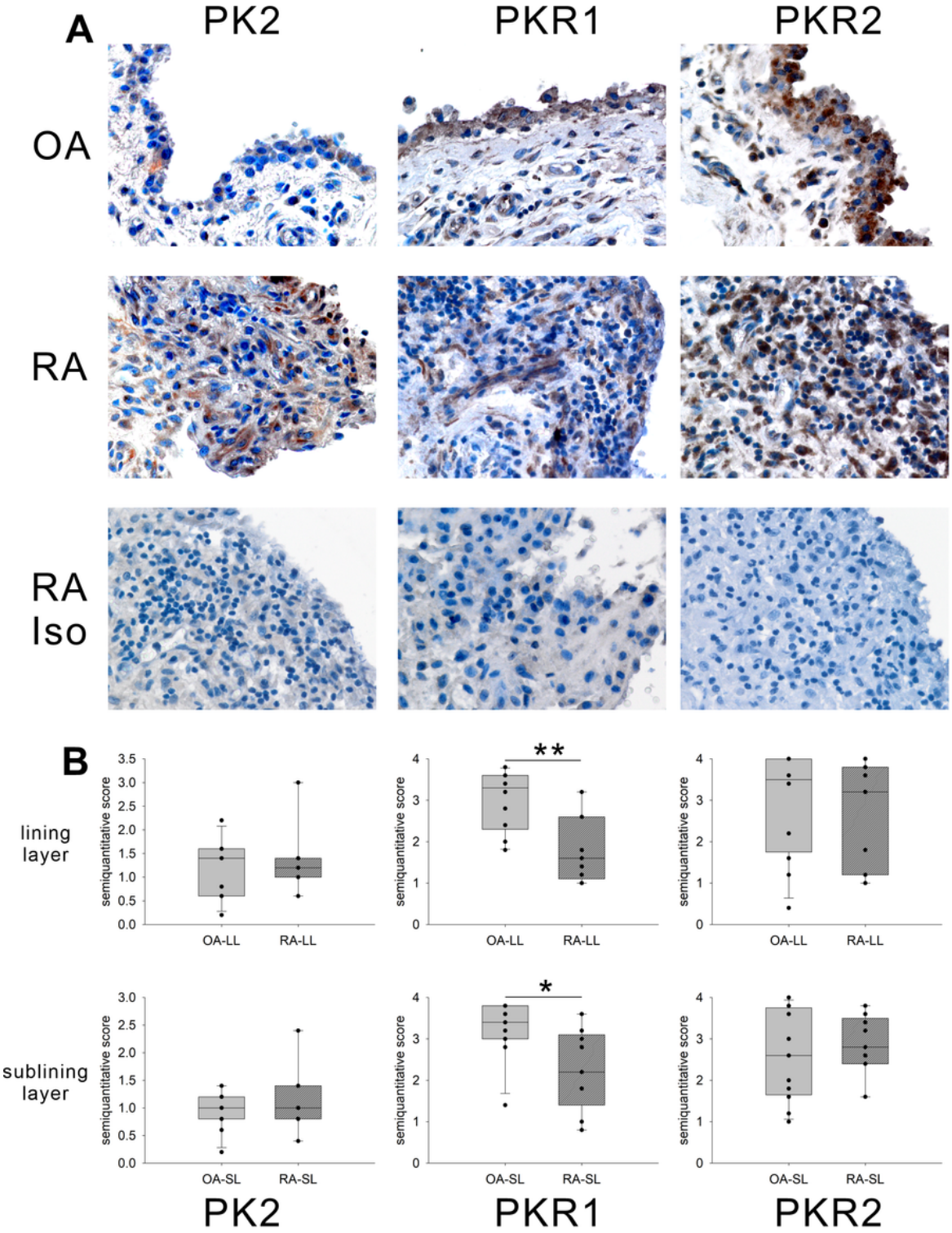

Figure 2. PK2, PKR1, and PKR2 expression in OA and RA synovial tissues.

Figure 2

PK2, PKR1, and PKR2 expression in OA and RA synovial tissues. (A) Immunostaining of PK2 (left panel), PKR1 (middle panel), and PKR2 (right panel) proteins in synovial tissue from OA and RA patients. Brown staining indicates PK2-, PKR1-, or PKR2-positive cells (×400) Staining with an isotype control as the

primary antibody in RA synovial tissue is also demonstrated. (B) Semiquantitative results of PK2, PKR1, and PKR2 expression. Semiquantification was performed using a scoring method (0–4) in the lining and sublining layers of synovial tissue. Data were presented as box plots, in which the boxes demonstrate the 25th and 75th percentiles, the lines within the boxes demonstrate the median, and the lines outside the boxes demonstrate the 10th and 90th percentiles. N = 8–10. For statistical analysis, the Mann–Whitney test was used. *p<0.05, **p<0.01. Abbreviations: PK2, prokineticin 2; PKR1, prokineticin receptor 1; PKR2, prokineticin receptor 2; OA, osteoarthritis; RA, rheumatoid arthritis; Iso, isotype control; SL, sublining layer; LL, lining layer.

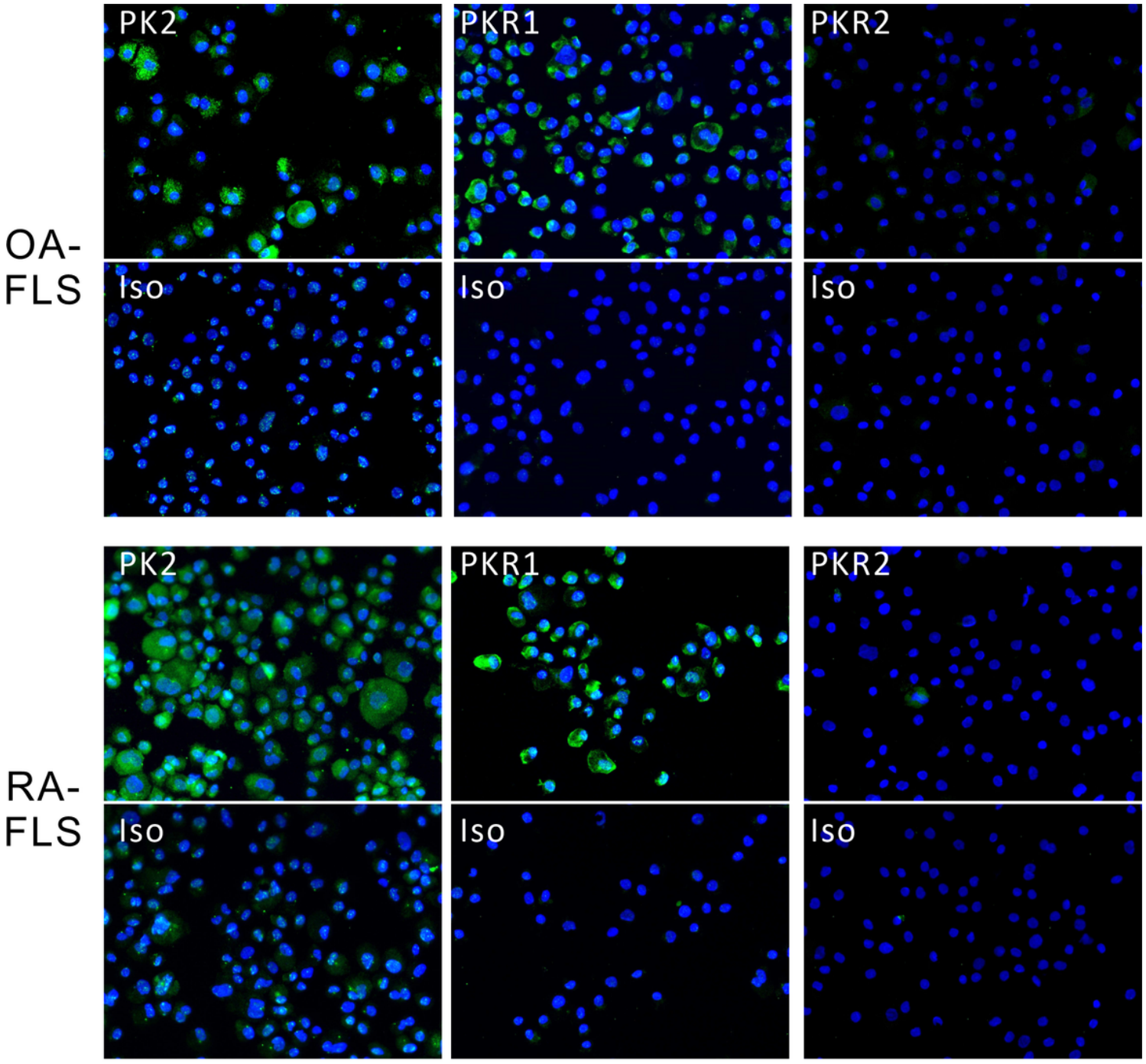

Figure 3. Immunofluorescent staining of PK2, PKR1, and PKR2 proteins in OA- and RA-FLS.

Figure 3

Immunofluorescent staining of PK2, PKR1, and PKR2 proteins in OA- and RA-FLS. Cells were subjected to cytospin. Green staining indicates PK2- (left panel), PKR1- (middle panel), or PKR2- (right panel) positive cells (×200). Stained sections with an isotype control as the primary antibody are also demonstrated. N = 5. Abbreviations: PK2, prokineticin 2; PKR1, prokineticin receptor 1; PKR2 prokineticin receptor 2; OA, osteoarthritis; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes.

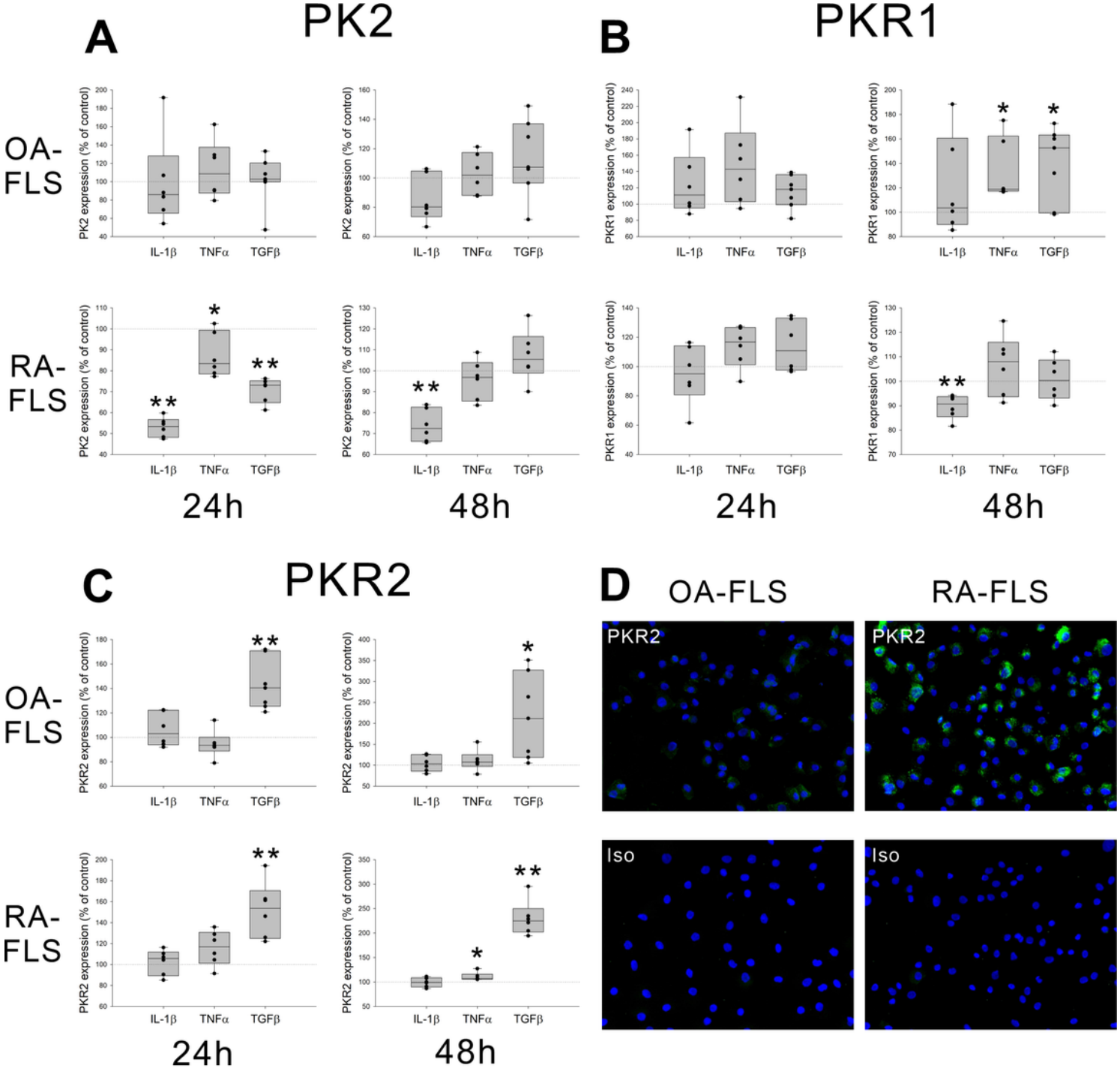

Figure. 4 Modulation of PK2, PKR1, and PKR2 expression in OA- and RA-FLS under proinflammatory conditions.

Figure 4

Modulation of PK2, PKR1, and PKR2 expression in OA- and RA-FLS under proinflammatory conditions. PK2 (A), PKR1 (B), and PKR2 (C) proteins in OA- and RA-FLS were examined by cell-based ELISAs at 24 and 48 h after stimulation with IL-1b (200 pg/ml), TNFa (10 ng/ml), and TGFb (10 ng/ml). (D) Immunofluorescent staining of PKR2 in OA- and RA-FLS at 48 h after stimulation with TGFb. N = 6. Green staining indicates PKR2-positive cells (×200). Staining with an isotype control as the primary antibody is also given. Data are shown as box plots, the description of which is given in the legend to Figure 2. For statistical analysis, the one-sample Wilcoxon signed-rank test (when normality was not given) or the one-sample t-test was used (when data were normally distributed). *$p<0.05$, **$p<0.01$. Abbreviations: PK2, prokineticin 2; PKR1, prokineticin receptor 1; PKR2, prokineticin receptor 2; OA, osteoarthritis; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; IL-1b, interleukin-1b; TNFa, tumor necrosis factor a; TGFb, transforming growth factor b; Iso, isotype control.

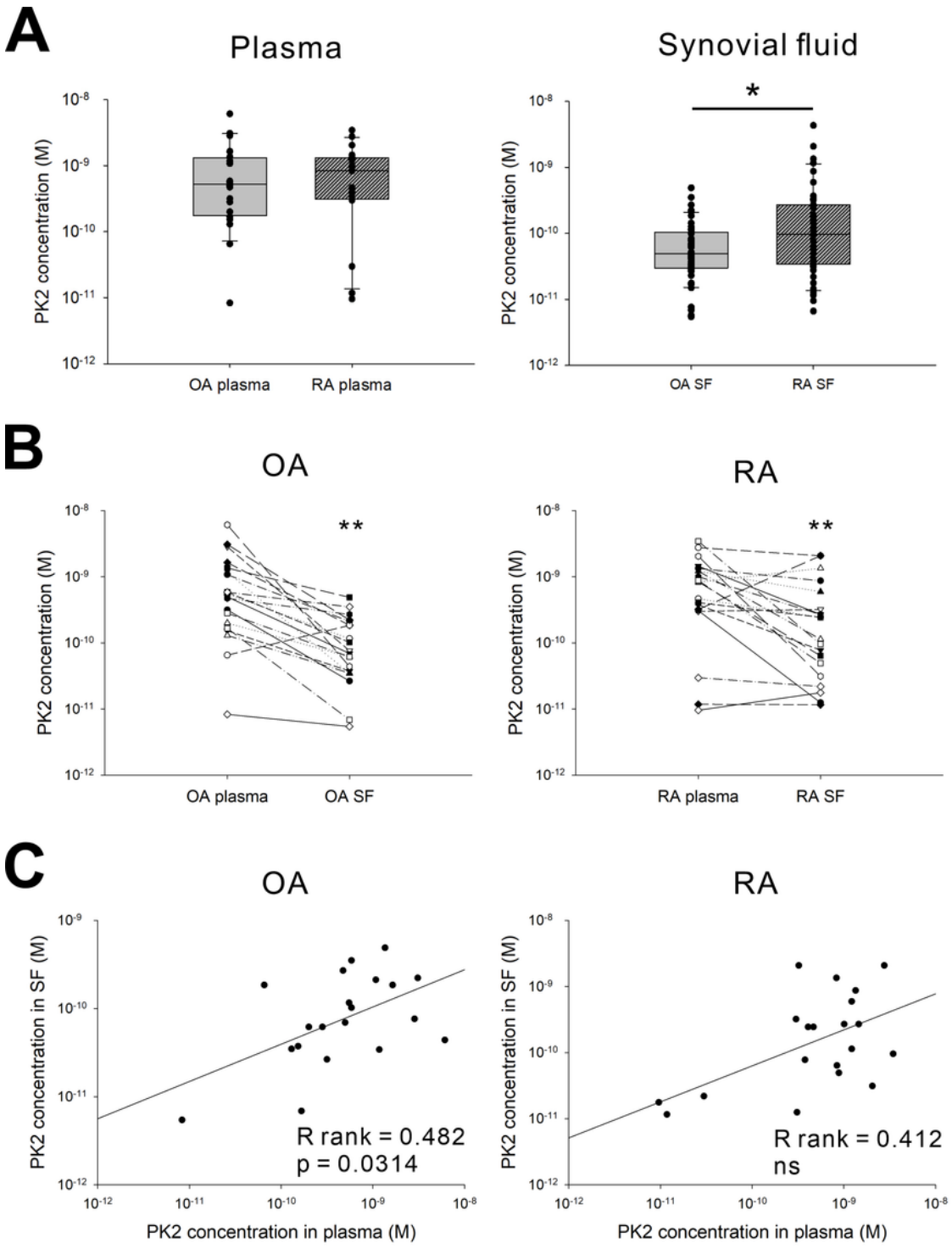

Figure 5. Figure 5. The PK2 concentration in plasma and synovial fluid from OA and RA patients.

Figure 5

The PK2 concentration in plasma and synovial fluid from OA and RA patients. (A) Comparison of plasma (N = 20) and synovial fluid (N = 50) PK2 concentrations between OA and RA patients. Data were presented as box plots, the description of which is given in the legend to Figure 2. For statistical analysis, the Mann–Whitney-test was used. *p<0.05, **p<0.01. (B) Comparison of PK2 concentrations between plasma and synovial fluid in OA and RA patients. N = 20. For statistical analysis, the Wilcoxon signed-

rank test was used. (C) Correlation of PK2 concentrations between plasma and synovial fluid in OA and RA patients. N = 20. The linear regression line, the Spearman rank correlation coefficient (R rank), and the respective p-value are given. Abbreviations: PK2, prokineticin 2; SF, synovial fluid; OA, osteoarthritis; RA, rheumatoid arthritis; NS, not significant.

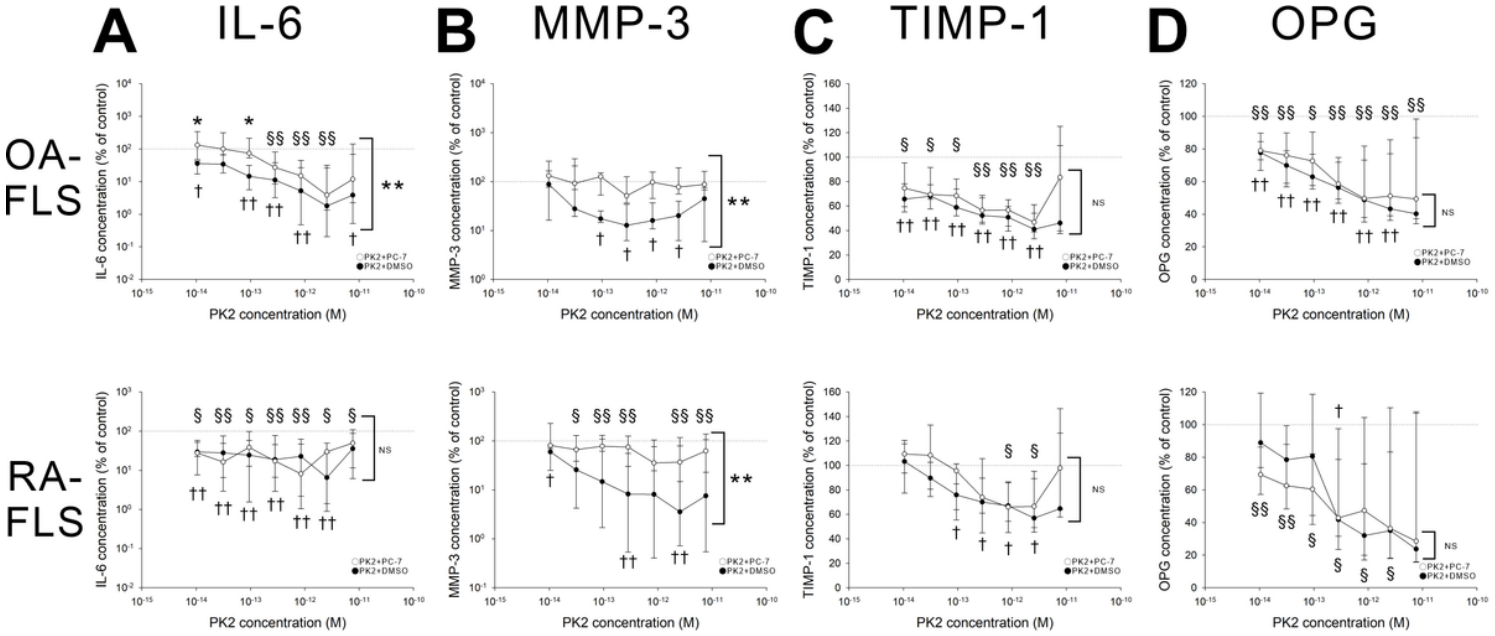

Figure 6. Influence of PK2 and PC-7 (or DMSO) on IL-6 (A), MMP-3 (B), TIMP-1 (C), and OPG (D) production from TNFα-prestimulated OA- and RA-FLS.

Figure 6

Influence of PK2 and PC-7 (or DMSO) on IL-6 (A), MMP-3 (B), TIMP-1 (C), and OPG (D) production from TNFa-prestimulated OA- and RA-FLS. The dotted line indicates the control level of 100% (TNFa without PK2). All data are given as the median (25th percentile, 75th percentile). N = 8. In comparisons between DMSO and PC-7 groups, a two-way ANOVA followed by the Bonferroni post hoc test was used (*p<0.05, **p<0.01). In comparisons with a control level of 100%, the one-sample Wilcoxon signed-rank test was used (compared with the control in the PC-7 group: §, p<0.05 and §§, p<0.01; compared with the control in the DMSO group: †p<0.05 and ††p<0.01). Abbreviations: PK2, prokineticin 2; DMSO, dimethylsulfoxide; OA, osteoarthritis; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; IL-6, interleukin-6; TNFa, tumor necrosis factor a; MMP-3, matrix metalloproteinase 3; TIMP-1, tissue inhibitor of metalloproteinase 1; OPG, osteoprotegerin; NS, not significant; ANOVA, analysis of variance.

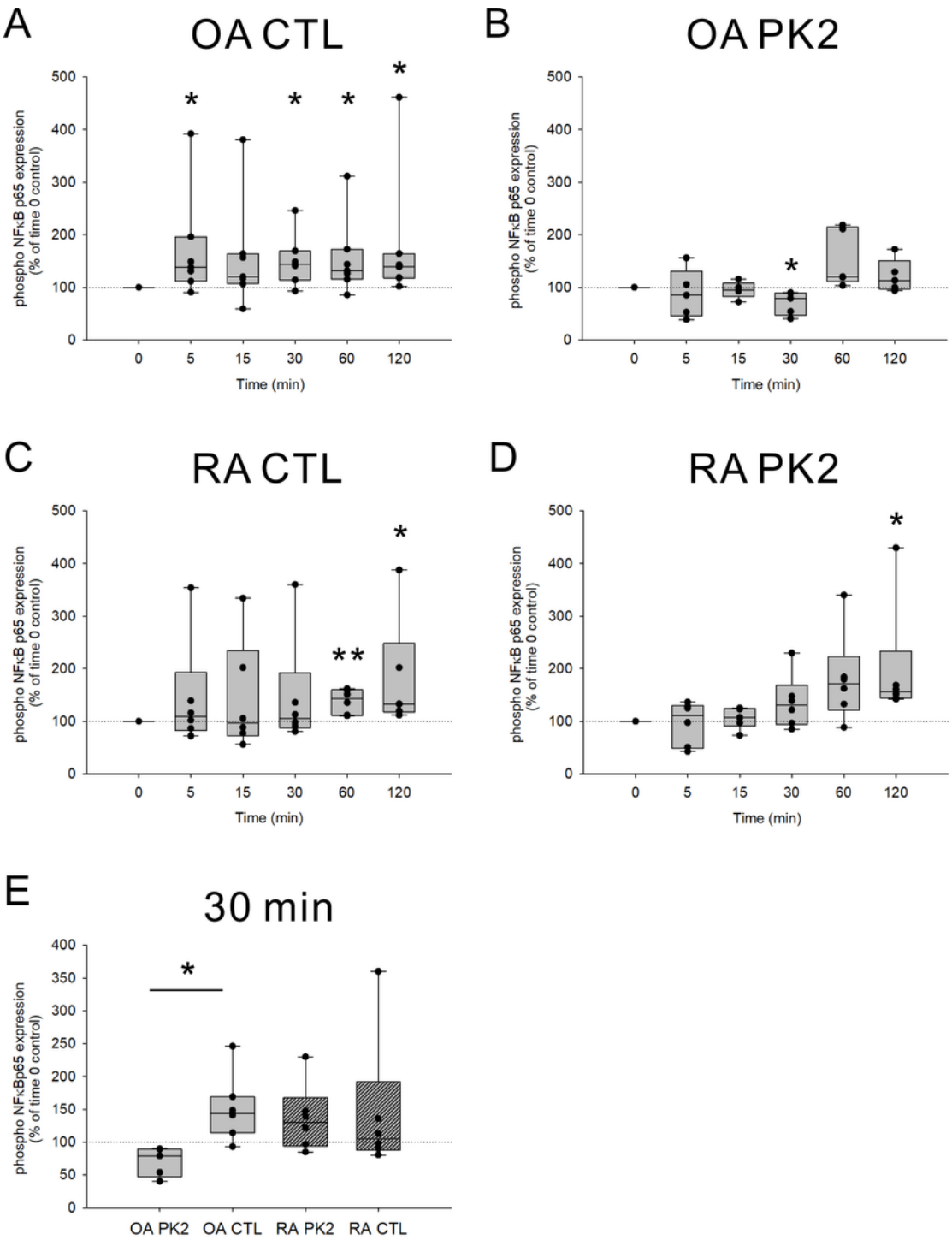

Figure 7. The induction of phospho-NFκB p65 by TNFα was attenuated in PK2 prestimulated OA-FLS.

Figure 7

The induction of phospho-NFkB p65 by TNFa was attenuated in PK2 prestimulated OA-FLS. Phospho-NFkB p65 protein in PK2 (10-11 M) or control (water) prestimulated OA- or RA-FLS was examined by cell-based ELISAs 0, 5, 15, 30, 60, and 120 min after stimulation with TNFa (10 ng/ml). (A) Control prestimulated OA-FLS. (B) PK2 prestimulated OA-FLS. (C) Control prestimulated RA-FLS. (D) PK2 prestimulated RA-FLS. (E) Comparisons of phospho-NFkB p65 expression between PK2 or control

prestimulated OA- or RA-FLS 30 min after stimulation with TNFa. N = 6. Data were given as box plots, the description of which is given in the legend to Figure 2. For statistical analysis, the one-sample Wilcoxon signed-rank test was used for comparisons with time 0 as the control, representing the 100% line. In comparisons among the groups at 30 min after stimulation with TNFa (E), the Mann–Whitney-test was used. *p <0.05, ** p<0.01. Abbreviations: NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; PK2, prokineticin 2; CTL, control; OA, osteoarthritis; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; TNFa, tumor necrosis factor a.

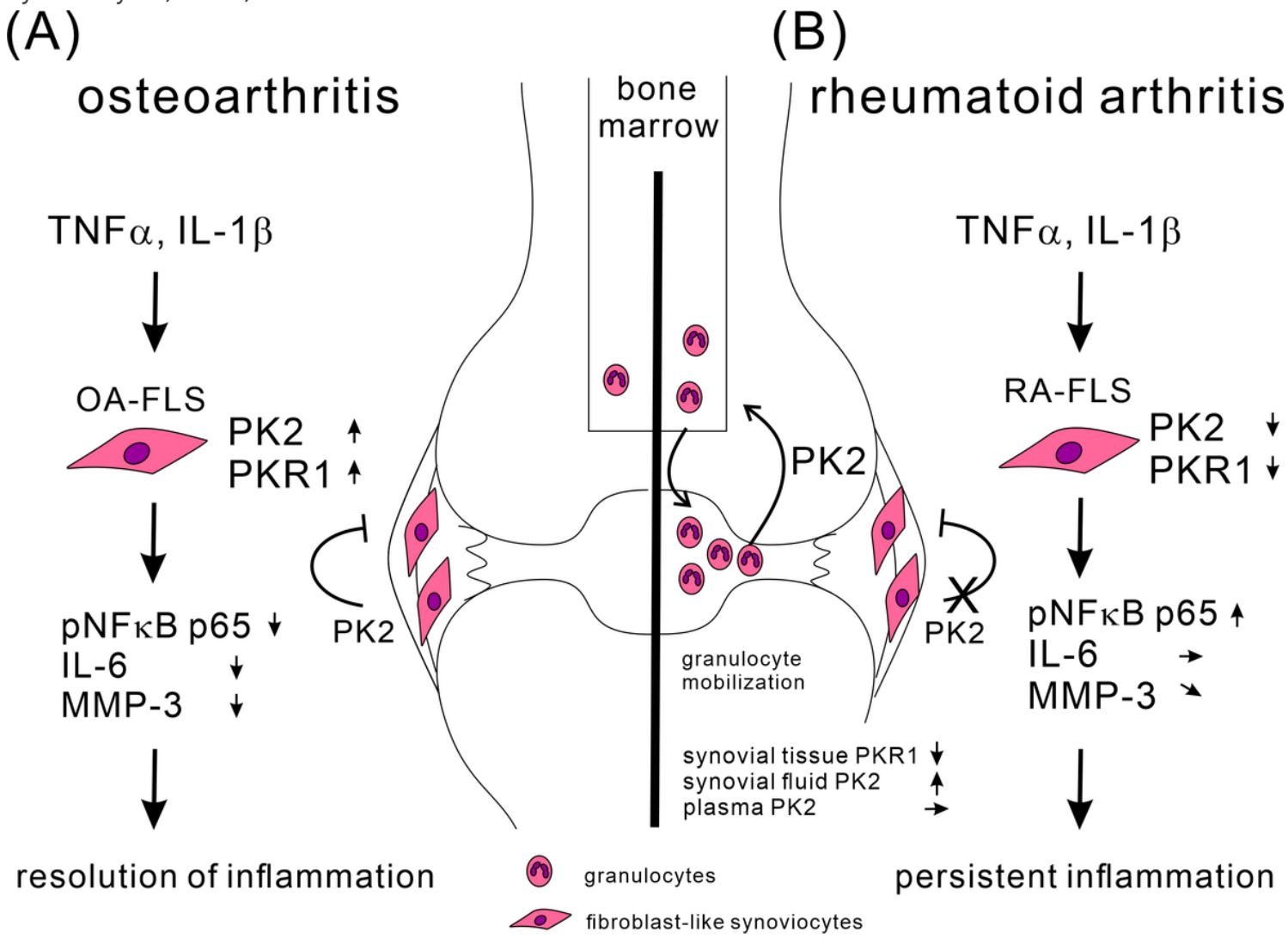

Figure 8. Model describing the dysregulation of endogenous inflammation by PK2 in RA.

Figure 8

Model describing the dysregulation of endogenous inflammation by PK2 in RA. In OA, exposure to proinflammatory cytokines upregulates the expression of PKR1. PK2 secreted from OA-FLS acts on PKR1 in an autocrine or paracrine manner and reduces the secretion of IL-6 and MMP-3 from OA-FLS (A). In contrast, in RA-FLS, exposure to proinflammatory cytokines downregulates the expression of PK2 and

PKR1 (B). Therefore, PK2 does not exhibit a similarly strong anti-inflammatory effect in RA-FLS compared with OA-FLS. Indeed, the expression of PKR1 in RA synovial tissue was decreased compared with OA synovial tissue. This dysregulation in the endogenous inflammation-mediated modulation of PK2 in RA-FLS may be associated with the chronicity of inflammation in the pathogenesis of RA. Abbreviations: PK2, prokineticin 2; PKR1, prokineticin receptor 1; OA, osteoarthritis; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; IL-1b, interleukin-1b; TNFa, tumor necrosis factor a; IL-6, interleukin-6; MMP-3, matrix metalloproteinase 3; pNFkB, phosphor nuclear factor kappa-light-chain-enhancer of activated B cells.

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