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CD73 blockade alleviates collagen-induced arthritis by inhibiting synovial fibroblast activity

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ABSTRACT

Objective(s): Rheumatoid arthritis (RA) is a chronic autoimmune disease with progressive cartilage erosion and joint destruction. CD73 plays a critical role in regulating inflammatory responses. This study aims to investigate the effects of CD73 blockade on RA progression and explore the potential mechanism.

Materials and Methods: Soluble CD73 levels were determined by enzyme-linked immunosorbent assay. Immunohistochemistry (IHC) was used to detect CD73 expression in tissues. A collagen-induced arthritis (CIA) rat model was used to evaluate the effect of CD73 inhibitors in vivo. Hematoxylin and eosin staining was used to evaluate synovial tissue inflammation. Safranin O/Fast Green staining and Micro-CT were used to evaluate cartilage erosion and bone destruction. A live cell imaging system was used to analyze cell proliferation. Cell invasion experiments were performed using Transwell chambers.

Results: Our data showed that CD73 expression was significantly increased in synovial tissues and serum from RA patients. The increased CD73 expression may be attributed to the hypoxic microenvironment via the hypoxia-inducible factor pathway in synovial tissues. Using CIA rats, our data demonstrated that CD73 blockade alleviated bone destruction and synovial inflammation. CD73 blockade inhibited synoviocyte proliferation, invasion, and pro-inflammatory cytokine production. Moreover, transcriptome analysis revealed that differentially expressed genes (DEGs) in synoviocytes induced by CD73 blockade were primarily enriched for inflammatory responses, neutrophil chemotaxis, and integrin-mediated signaling pathways.

Conclusion: CD73 blockade alleviated RA disease progression by regulating the activity of synovial fibroblasts. CD73 blockade is a potential therapeutic approach for patients with RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease with unclear etiology characterized by inflammatory synovial hyperplasia, joint stiffness, progressive bone erosion, and systemic manifestations (1). During RA occurrence and progression, synovial inflammation is a critical driving factor. Synovial fibroblasts (SFs) are critical in driving synovial inflammation, at least in part via secreting inflammatory cytokines (2, 3). Thus, the molecules that regulate SF activation are critical to RA development.

CD73 (ecto-5'-nucleotidase), encoded by the NT5E gene, catalyzes the conversion of AMP to adenosine and plays an important role in human immune regulation. CD73 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein that can be shed from the cell membrane, enter the circulation, and exist as a soluble form (4). Notably, serum soluble CD73 (sCD73) levels are elevated in multiple diseases and correlate with disease activity, progression, or prognosis, including melanoma and pancreatitis (5, 6).

To date, accumulating evidence has demonstrated that CD73 up-regulation promotes cancer progression (7, 8). Importantly, CD73 has been considered a promising therapeutic target for cancer, and multiple animal experiments and clinical trials have presented the therapeutic efficacy of CD73 blockade (9).

Beyond cancer, given CD73's critical role in immune regulation, several studies have investigated its role in autoimmune diseases, including systemic lupus erythematosus (10), experimental autoimmune encephalomyelitis (11), and RA (12, 13). Chrobak *et al.* demonstrated that CD73 deficiency in C57BL/6 mice increased susceptibility to Collagen-induced arthritis (CIA). CIA is a commonly used animal model of RA that can be performed in rats or mice. However, the role of CD73 blockade in RA progression remains unclear. In this study, we investigated the effects of CD73 blockade on RA disease and synovial fibroblast activation, and further explored the underlying mechanisms.

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Materials and Methods

Synovial tissue and synovial fibroblasts

The studies involving human participants were approved by the Ethics Committee of Tangdu Hospital, Fourth Military Medical University (202303-154). The specimens used in this study were not individually identifiable or traceable, and the waiver of informed consent would not have a negative impact on the rights and interests of the subjects. The informed consent waiver was approved by the Ethics Committee of Tangdu Hospital, Fourth Military Medical University, in accordance with the (Approaches to ethical review of biomedical research involving humans) by the State Health and Family Planning Commission, China.

Synovial tissues were obtained from patients with RA (n=3) or OA (n=3) who underwent joint replacement surgery performed by the Department of Orthopedics, Tangdu Hospital. All RA and OA patients satisfied the clinical and radiographic criteria of the American College of Rheumatology.

SFs were isolated from synovial tissues according to a previously described method (14). Fresh synovial tissues were minced and digested with 4 mg/ml type I collagenase in DMEM at 37 ˚C for 2 hr. Next, the cell suspensions were filtered through a 75 μm disposable nylon mesh under gravity. Then, SFs in the filtered supernatant were collected by centrifugation and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; ExCellBio, Shanghai, China) and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA) at 37 ˚C with 5% CO_2 in a humidified incubator. To ensure the biological activity of the SFs, cells used in all experiments were at passages 3-5. The purity of RA synovial fibroblasts (RASFs) was verified by flow cytometry (FCM). Cells were stained with FITC-conjugated anti-CD68 (Cat: FHF068, 4A Biotech Co., Ltd, Beijing, China) and PE-conjugated anti-CD90 (Cat: FHP090, 4A Biotech Co., Ltd, Beijing, China) antibodies. $CD68^-CD90^+$ cells were defined as RASFs.

Serum collection

The peripheral blood samples from OA (n=74) and RA (n=80) patients were collected, with healthy subjects (n=71) serving as the control group. Serum was isolated by centrifugation and stored at -70 ˚C. The clinical characteristics of RA patients are presented in Table 1 (Supplementary Material).

Cell culture

The MH7A cell line was cultured in DMEM supplemented with 10% FBS (ExCellBio, Shanghai, China) in a humidified incubator at 37 ˚C with 5% CO_2. Hypoxic culture conditions were established using an automated anaerobic cultivation system (Anoxomat, MART Microbiology B.V., Netherlands) under hypoxic conditions (0.1 % O_2, 24 hr, 48 hr). The Anoxomat system precisely regulated the gas environment via a gas displacement vacuum method; residual oxygen was further depleted using palladium particle catalysts to achieve the hypoxic conditions required for the experiments.

Detection of CD73, IL-6, and IL-8

Soluble CD73 concentration in serum was detected by using a CD73 DuoSet ELISA kit (CAT: DY5795-05, R&D, USA). The secretion of IL-6 by human RASFs was measured based on the electrochemical luminescence assay (Cat: (10)72138101, Roche, Switzerland) adapted to an automated chemiluminescent analyzer (Cobas e801, Roche, Switzerland). IL-8 production from human RASFs was detected by using an ELISA kit (R&D Systems, MN, USA). IL-6 and IL-8 levels in the lysate of synovial tissue from CIA-rats were detected by using ELISA kits (A&E Bio, Shaanxi, China). All experimental procedures were in accordance with the manufacturer's instructions.

Detection of CRP, ESR, RF, anti-CCP, and ASO

The concentrations of serum C-reactive protein (CRP), rheumatoid factor (RF), and antistreptolysin O (ASO) were measured using an automated protein analyzer (BN II System, SIEMENS, Germany). The serum anti-CCP antibody levels were measured via an enzyme-linked immunosorbent assay (Kexin Biotech, Shanghai, China). Erythrocyte sedimentation rate (ESR) was determined by measuring erythrocyte sedimentation within 1 hr using an ESR analyzer (Monitor-100, Italy). Results are reported as millimetres per hour (mm/h) according to the Westergren method.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (GLPBIO, Montclair, CA, United States) according to the manufacturer's protocol. Genomic DNA contamination was removed, and cDNA was generated by using a reverse transcription kit (Cat: AG11702, Accurate Biotechnology, Hunan, China). Briefly, the genomic DNA removal step was performed at 42 ˚C for 2 min on an ice-prepared reaction system, followed by reverse transcription reaction at 37 ˚C for 15 min, enzyme inactivation at 85 ˚C for 5 s, and final hold at 4 ˚C. qRT-PCR was performed by using BlasTaq™ 2X qPCR Master Mix with SYBR Green chemistry (Cat: G891, Abm, Jiangsu, China). The PCR amplification conditions were 95 ˚C for 3 min, 40 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min, followed by melting curve analysis. The relative expression of mRNA was calculated by the ΔCt values using the Mann-Whitney U test, with GAPDH used as the internal reference. The primers were listed in Table 1.

The IHC assay was used to evaluate the protein levels in synovial tissues. Synovial tissues were prepared as formalin-fixed paraffin-embedded (FFPE) sections. In brief, anti-CD73 antibody (1:200 dilution, Abcam ab175396) and anti-HIF-1α (1:100 dilution, Abcam ab16066) were used as the primary antibodies. The sheep anti-rabbit (1:200 dilution, Servicebio GB23303) and sheep anti-mouse (1:200 dilution, Servicebio GB23301) were used as secondary antibodies. Pannoramic and CaseViewer 2.4 software (3DHISTECH, Hungary) were used for image acquisition and analysis.

Collagen-induced arthritis (CIA) rat model

Sprague-Dawley (SD) rats (6 weeks; Female; 180-200g; n=21) were purchased from the Animal Experiment Center, Air Force Medical University. Rat experiments were performed according to animal welfare guidelines approved by the Air Force Medical University's Institutional Animal Care and Use Committee (No.20230837).

The CIA model was established as described previously (15). 21 SD rats were randomly divided into the control group (n=5) and the CIA model group (n=16). In short, rats were injected intradermally with an emulsion (chicken type II collagen+complete Freund's adjuvant (CFA)) into

Table 1. Primer sequences used for qRT-PCR

Gene	Forward (5' - 3')	Reverse (5' - 3')
Human		
GAPDH	TCCTTGGAGGCCATGTGGGCCAT	TGATGACATCAAGAAGGTGGTGAAG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
CD73	GCCTGGGAGCTTACGATTTTG	TAGTGCCCTGGTACTGGTCG
HIF-1α	TCCATCTCCTACCCACATACA	CTCAAAGCGACAGATAACACG
Rat		
GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT
IL-6	ACTTCCAGCCAGTTGCCTTCTTG	TGGTCTGTTGTGGGTGGTATCCTC
IL-8	ATGACTTCCAAGCTGGCCGTGG	TGAATTCTCAGCCCTCTTCAAAAAC

the tail base, followed by a booster immunization 7 days later. The control rats were only injected with CFA. CIA progression was monitored by scoring the paws from 14 days after immunization. Each paw was graded from 0 to 4: 0=normal; 1=erythema and mild swelling; 2=erythema and swelling extending to ankle joints and one or two toes; 3=erythema and swelling extending to metatarsal joints and more than two toes; and 4=ankylosing deformity with joint swelling. The arthritic score was equal to the sum of four paw scores. Rats with an arthritic score >2 were selected as the CIA model.

CIA model group rats were randomly divided into the CIA group and the APCP treatment group. APCP (M3763, Sigma, USA) was administered at 4 mg/kg via intraperitoneal injection (i.p.) once every 4 days for 3 weeks in CIA rats. Phosphate buffer solution (PBS) was administered via i.p. injection as the CIA+PBS group. Arthritis severity in rats was assessed every 4 days from day 14 after immunization. On day 34, the rats were sacrificed under isoflurane inhalation anesthesia, and the bilateral ankle joints of the rats were removed to maintain the integrity of the ankle joints for subsequent analysis.

Histopathological analysis

Rat ankle joints were stored in 4% formaldehyde, decalcified in 0.5 M ethylenediaminetetraacetic acid (EDTA) for 8 weeks. Then, FFPE sections of rat ankle joints were prepared. Then, Hematoxylin and eosin (H&E) staining and Safranin O/Fast Green staining were used to evaluate synovial tissue inflammation and articular cartilage destruction. Pannoramic and CaseViewer 2.4 software (3DHISTECH, Hungary) were used for image acquisition and analysis.

Micro-CT analysis

The right ankle joints of rats were dissected for micro-CT measurement using the Perkin Elmer Quantum GX microCT Imaging System (Waltham, MA, USA). Three-dimensional reconstruction was performed using the volume rendering method, and the bone mineral density (BMD) was calculated using built-in software.

Cell proliferation and invasion experiment

RASFs were seeded in 96-well plates with a density of 2×10^3 cells/well and cultured at 37 ˚C with 5% CO_2 in a live cell imaging system (CYTATION I, BIOTECK). The cells were imaged every four hours. There were 6 replicate wells in each group. Cell invasion experiments were performed using Transwell chambers (Corning, NY, USA). Cells were added to the upper Matrigel-coated chambers and inserted into 24-well plates in DMEM containing 20% FBS. After incubation at 37 ˚C for 48 hr, the filters were fixed with 75% ethanol for 10 min and stained with 0.1% crystal violet for 30 min. A cotton swab was used to gently remove cells from the upper surface of the membrane. Then, 5 randomly selected fields in each well were photographed and counted.

Transcriptome sequencing

The total RNA from RASFs and APCP-treated RASFs was extracted. Library preparation and sequencing were performed on the Illumina platform using paired-end 150-bp reads by Lianchuan Bio, China. Gene expression levels were represented by fragments per kilobase of transcript per million fragments mapped (FPKM). The sequencing data were submitted to the Sequence Read Archive (SRA) of NCBI. The accession number was SRR25387777.

The expression levels with $|Log_2FC|\geq1$ (FC: fold change) and $P<0.05$ were screened as the Differentially Expressed Genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for the DEGs were performed. R-bubble diagrams were used to visualize enriched GO terms and KEGG pathways.

Statistical analysis

The Kolmogorov-Smirnov test was used to check the normal distribution of the data. Normally distributed quantitative data were presented as mean±standard deviation (SD) and analyzed by Student's t-test. Non-normally distributed data were presented as median (interquartile range, IQR) and analyzed by the Mann-Whitney U test. Spearman's rank correlation was used to assess the association between indices. Repeated-measurement data were analyzed by repeated-measures ANOVA combined with a Bonferroni *post hoc* test. All analyses were performed using GraphPad Prism 8.0 software. $P<0.05$ was considered to be statistically significant.

Results

CD73 was increased in serum and synovial tissue from rheumatoid arthritis patients

We investigated CD73 expression levels in synovial tissues and serum from RA patients. First, the IHC results showed that CD73 levels were higher in synovial tissues from RA patients than in those from OA patients (Figure 1A). Furthermore, RASFs were isolated from RA synovial tissues and identified by flow cytometry (Figure 1B). CD73 mRNA levels were higher in RASFs than in OASFs (Figure 1C). Moreover, compared with healthy subjects and OA patients, the serum CD73 levels were also higher in RA patients (Figure 1D). Notably, serum CD73 levels were positively correlated with serum CRP, ESR, and RF levels in RA patients (r=0.296, *P*=0.015; r=0.264, *P*=0.031; r=0.269, *P*=0.038; Figure1E-I). There was a significant positive correlation between serum CD73 and disease activity of RA patients, which was indicated by DAS28-ESR (Figure 1J; r=0.348, *P*=0.030) or DAS28-CRP scores (Figure 1K; r=0.359, *P*=0.029).

CD73 blockade alleviates bone erosion and synovial inflammation in RA rats

APCP, a specific CD73 inhibitor (16), was administered via intraperitoneal injection to CIA-rats, and the effect of APCP treatment on RA disease progression was evaluated. The schematic of rat experiments was shown in Figure 2A. Compared to CIA-rats, arthritis scores in APCP-treated CIA rats were significantly decreased (Figure 2B). Micro-CT showed that APCP treatment alleviated bone destruction in CIA-rats (Figure 2C). The total BMD was higher in APCP-treated CIA rats than in control CIA rats (Figure 2D). Furthermore, H&E and safranin O staining showed that APCP reduced synovial inflammation and cartilage erosion in CIA rats (Figures 2E and 2F).

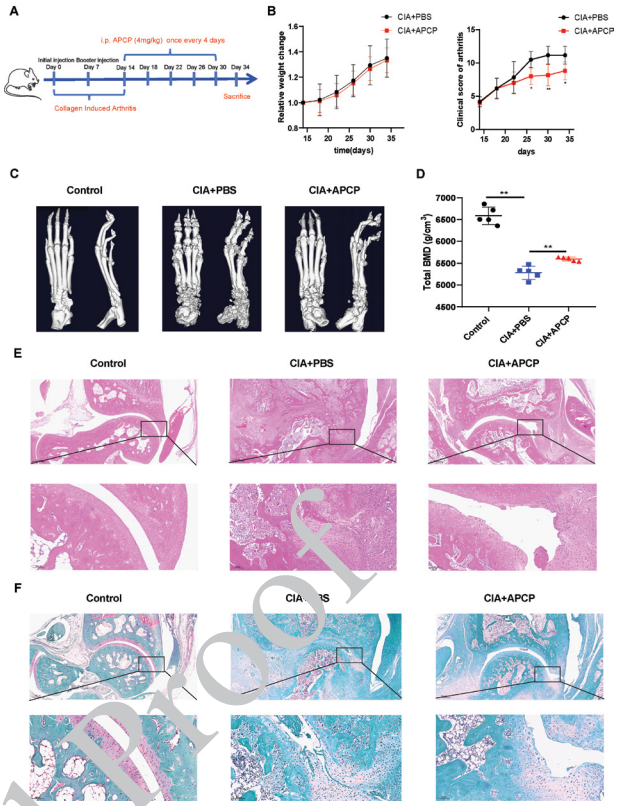

Figure 2. Adenosine 5′-(α,β-methylene) diphosphate sodium salt (APCP) alleviated the disease severity in collagen-induced arthritis (CIA) rats
(A) The flow diagram of CIA rat experiments. (B) The effect of APCP treatment on body weight and arthritis scores in CIA rats. (C) Micro-CT showing the effect of APCP treatment on bone destruction in CIA rats. (D) The effects of APCP treatment on the bone mineral density (BMD) of CIA rats. (E) Hematoxylin-eosin (H&E) staining was performed to assess synovial inflammation in rats of each group. Scale bar=500 μm; scale bar=100 μm. (F) Safranin O/Fast green staining was assessed to evaluate cartilage damage in each group of rats. Scale bar=500 μm; scale bar=100 μm. n=5 rats per group

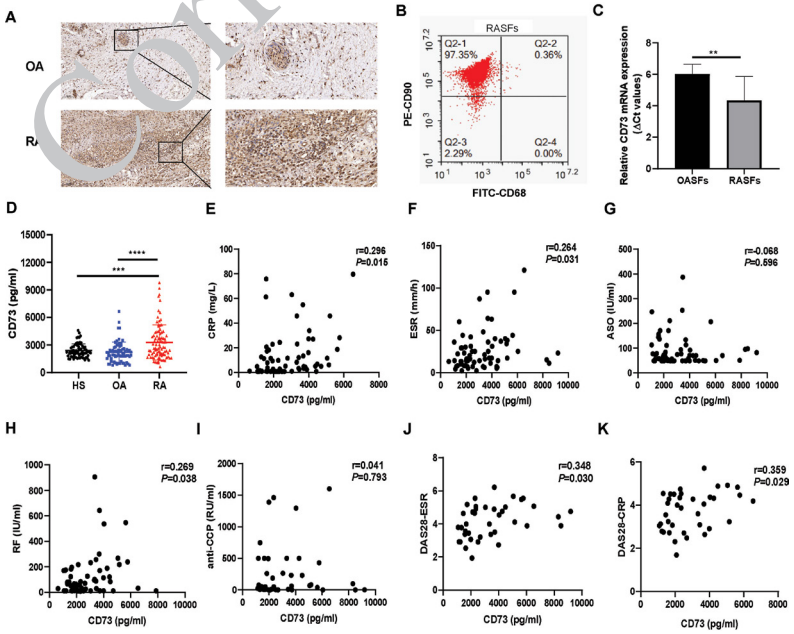

Figure 1. CD73 was highly expressed in rheumatoid arthritis (RA) and positively correlated with disease activity
(A) The expression level of CD73 in RA synovial tissue was higher than that in OA synovial tissue (n=3). (B) Identification of primary synovial fibroblasts by flow cytometry. (C) CD73 expression levels were higher in RASFs than in OASFs (n=3). Statistical significance was assessed by the Mann-Whitney U test. (D) Data are from the independent serum cohort. Serum CD73 levels were higher in RA patients (n=80) than in healthy subjects (n=71) and OA patients (n=74). Statistical significance was assessed by Student's t-test. (E-I) The correlation between serum CD73 levels and CRP, ESR, ASL, RF, and anti-CCP. (J, K) The correlation between serum CD73 levels and DAS28-ESR and DAS28-CRP. Spearman's rank correlation was used to assess the association between indices

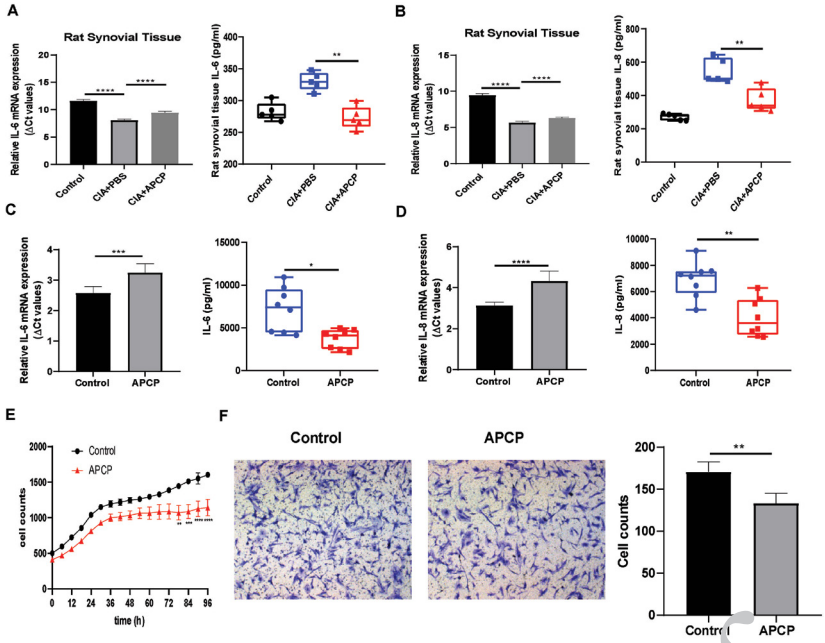

Figure 3. APCP treatment inhibited IL-6 and IL-8 expression in rats
(A, B) The mRNA and protein expression of IL-6 and IL-8 in the synovial tissues of CIA rats. n=5 rats per group. Statistical significance was assessed by the Mann-Whitney U test. (C, D) The effect of APCP treatment on IL-6 and IL-8 levels in RASFs. n=8. Statistical significance was assessed by Student's t-test. (E) The effect of APCP treatment on RASFs proliferation. n=6. Statistical significance was assessed using repeated-measures ANOVA followed by a Bonferroni post hoc test. (F) The effect of APCP treatment on RASFs invasion. Scale bar = 200 μm. n=5. Statistical significance was assessed by the Mann-Whitney U test

CD73 blockade inhibited the activity of RASFs

We next assessed the effect of CD73 blockade on RASF activity. First, compared with CIA rats, the mRNA and protein levels of pro-inflammatory cytokines (IL-6 and IL-8) in synovial tissues from APCP-treated CIA rats were significantly lower (Figures 3A and 3 B). Consistently, *in vitro* APCP treatment significantly inhibited IL-6 and IL-8 mRNA expression and secretion (Figures 3C and 3D). Second, Cell proliferation experiments and the Transwell assay showed that APCP treatment significantly inhibited RASF proliferation and invasion (Figures 3E and F).

HIF-1α induced the up-regulation of CD73 in RA

There was a hypoxia response element (HRE) contained in the promoter region of the CD73-encoding gene. Thus, mechanistically, HIF-1α transcriptionally up-regulated CD73 expression. Compared with OA patients, the mRNA and protein expression of HIF-1α were increased in synovial tissues from RA patients (Figure 4A, B). Next, we investigated the change of CD73 expression in the MH7A cell line under hypoxic culture conditions (0.1% O_2). As shown in Figure 4C, hypoxia significantly up-regulated the CD73 mRNA expression in MH7A. Compared with normoxia, the expression of HIF-1α, IL-6, and IL-8 in MH7A was significantly increased under hypoxia (Figure 4D-F). In addition, APCP treatment significantly inhibited the expression of IL-6 and IL-8 under hypoxic conditions (Figures 4G and H).

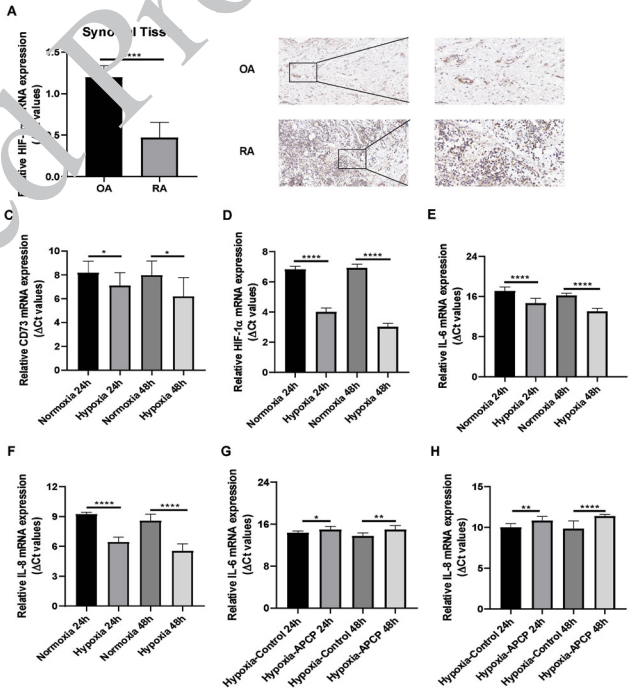

Figure 4. Hypoxia induced CD73 expression in MH7A
(A, B) HIF-1α expression was increased in rheumatoid arthritis (RA) synovial tissues (vs OA synovial tissues). (C) CD73 expression levels were up-regulated in MH7A cells under hypoxic conditions (0.1% O2). (D-F) The effect of hypoxic culture on HIF-1α, IL-6, and IL-8 levels in MH7A cells. (G, H) The effect of APCP treatment on IL-6 and IL-8 levels in MH7A under hypoxic conditions. n=3. Statistical significance was assessed by the Mann-Whitney U test

Transcriptomic Sequencing of APCP-treated RASFs

To explore the potential mechanism underlying CD73 blockade's effect on RASF activation, transcriptomic sequencing was performed (RASFs vs APCP-treated RASFs). There were 155 up-regulated and 19 down-regulated genes in APCP-treated RASFs (Figure 5A, B; Supplementary Material Table 2). Gene Ontology biological processes (GO_BP) analysis showed that these differentially expressed genes (DEGs) were mainly enriched in multiple biological processes, including inflammatory response, neutrophil

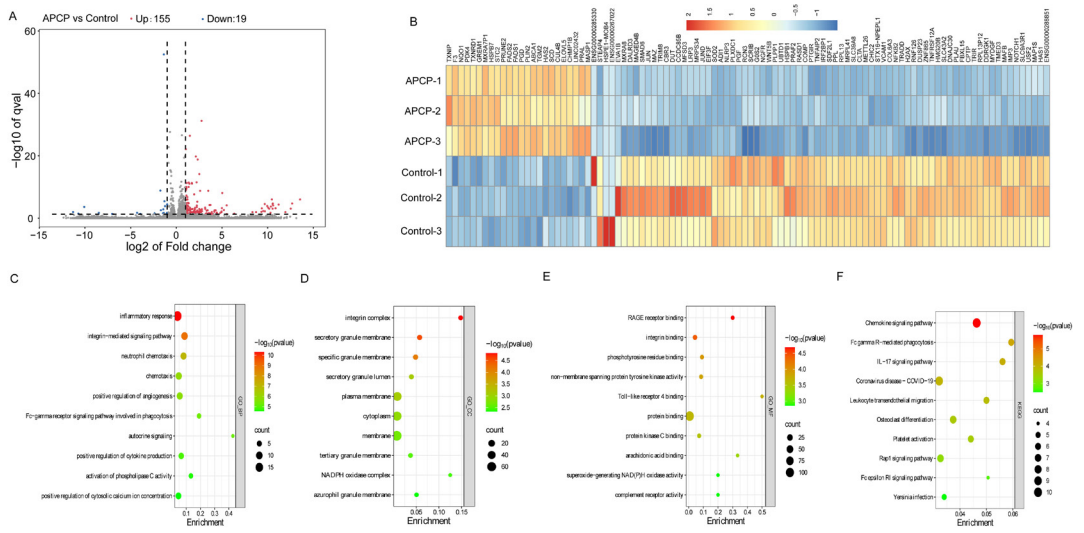

Figure 5. Transcriptomic analysis for the effect of APCP treatment on rheumatoid arthritis synovial fibroblasts (RASFs)
(A, B) Volcano plot and heat map showed the differentially expressed genes (DEGs) in APCP-treated RASFs (vs control RASFs). (C-E) GO enrichment analysis of the DEGs. (F) KEGG pathway analysis of the DEGs

chemotaxis, integrin-mediated signaling pathways, positive regulation of angiogenesis, and autocrine signaling (Figure 5C). GO cell component (GO_CC) analysis showed the DEGs were enriched in granule membrane, plasma membrane, and cytoplasm (Figure 5D). GO molecular function analysis showed the DEGs were mainly associated with protein binding (Figure 5E). KEGG analysis showed that these genes were associated with the immune system, including the chemokine signaling pathway, IL-17 signaling pathway, and Fc gamma R-mediated phagocytosis (Figure 5F).

Discussion

In this study, we found that CD73 expression level were significantly increased in synovial tissues, SFs, and serum from RA patients. Notably, the serum CD73 levels were positively correlated with RA disease activity. More importantly, we showed that CD73 blockade could alleviate disease activity in CIA rats, including bone destruction, synovial inflammation, and cartilage erosions, indicating that CD73 could be a potential therapeutic target for RA patients.

Firstly, why are the CD73 levels increased in RA synovial tissues? Hypoxia arises from increased cellular oxygen demand during the inflammatory response. Previous studies have shown that HIF-1α is increased in synovial tissues due to a hypoxic microenvironment, findings consistent with the present study (17). HIF-1α has been regarded as a potential therapeutic target for RA patients (18). Notably, since a hypoxia response element is contained in the CD73 promoter region, we investigated the effect of oxygen deficit on CD73 expression in the RASF cell line, MH7A. Our results demonstrated that the increased CD73 expression could be induced by the oxygen deficit in MH7A.

Secondly, is increased CD73 merely a concomitant phenomenon during RA development, or does it contribute to RA progression? By using CIA rats, a classical model for RA research, we found that CD73 blockade alleviated the RA disease activity in CIA rats, including reduced bone damage and synovial inflammation. Moreover, our results showed that CD73 blockade could inhibit the proliferation, invasion, and expression of inflammatory cytokines of RASFs. Taken together, these data indicate that CD73 may contribute to RA pathogenesis. This conclusion could be supported by the positive correlation between serum CD73 levels and DAS28-CRP of RA patients. Thus, for a molecule with altered levels in a particular disease, both the potential biomarker value and its role (promote or protect) in disease progression need to be explored. For example, CRP is a non-specific inflammatory indicator and is often used to assess disease activity in RA patients. Besides being a biomarker, Liang *et al.*'s study has shown that CRP has a detrimental effect in CIA rats. Inhibition of CRP using small interfering RNA or an antibody could attenuate bone erosion (19). Here, the positive correlation between serum CD73 and CRP levels points to a potential link between CD73 up-regulation and heightened inflammatory activity in RA.

However, Chrobak *et al.*'s report showed that CD73-deficient C57BL/6 mice were significantly more susceptible to CIA than wild-type mice, indicating a protective role of CD73 in collagen-induced arthritis (13). This seems to contradict the conclusions of our study. Regarding Chrobak *et al.*'s study, CD73 is manipulated prior to arthritic induction in genetic models (CD73-knockout mice). For the present study, CD73 was blocked after arthritic induction in CIA rats. Thus, these results might reveal the different roles of CD73 in distinct stages of RA development (i.e., early induction stage or disease occurrence versus disease progression). Actually, previous studies also found that CRP plays opposite roles in the initiation and progression of RA (20, 21). In other diseases, the opposite roles of certain molecules in distinct stages were also reported. For example, Yang *et al.* showed that mitochondrial transcription factor A (TFAM) plays opposing roles in the initiation and progression of colitis-associated cancer (22). In summary, CD73 may serve as a potential protective factor in the early phase of RA initiation, but plays a pathogenic role during active RA. Therefore, CD73 blockade might be used for therapy but not for RA prevention.

More importantly, CD73 catalyzes the conversion of extracellular AMP to adenosine and exerts its physiological

function by binding to G protein-coupled adenosine receptors (A_1R, A_2AR, A_2BR, and A_3R) on SFs and immune cells (23). A2A receptor activation can increase cAMP levels, inhibit NF-κB signaling pathways, reduce the secretion of TNF-α, IL-1β, IL-6, and other pro-inflammatory factors, and alleviate cartilage erosion. Activation of A_2B receptors might promote osteoclast activity and aggravate joint destruction (24,25). These results suggest that the dual effects of CD73 in RA may also stem from the activation of distinct subtypes of adenosine receptors.

During RA progression, SFs switch to invasive tumor-cell-like properties, which promote synovial inflammation and bone destruction. In this study, we found that CD73 was more highly expressed in SFs from RA than those from OA patients. Accumulated data indicate that CD73 can affect the biological behavior of multiple cell types, including tumor and immune cells (26-28). However, the effect of CD73 on RASF behaviors was unclear. Our results showed that CD73 blockade could inhibit the proliferation, invasion, and inflammatory cytokines production. These results could explain the alleviation of CIA rats by CD73 blockade.

The present study had some limitations. Firstly, as a competitive inhibitor, APCP has potential off-target effects on other ecto-nucleotidases involved in purine signaling. Secondly, APCP is susceptible to ionization at physiological pH, resulting in a short half-life *in vivo* and poor permeability of joint tissues. Finally, we only focused on the effect of the CD73 inhibitor on the phenotype of SFs, but did not explore the effects on other cells (e.g., immune cells, endothelial cells) and the mechanism of the downstream signaling cascade. In future studies, specific CD73 inhibitors or adenosine receptor inhibition assays will be used to further validate the mechanism of CD73 involvement in RA based on the RNA-sequencing analysis.

Conclusion

We demonstrated that CD73 expression was increased in RA patients and positively correlated with disease activity. CD73 blockade alleviates RA disease activity by inhibiting the proliferation, invasion, and cytokine production of RASFs. These data suggest that CD73 may represent a therapeutic target for RA patients.

Acknowledgment

This research was funded by the Science and Technology Innovation Development Fund of the Second Affiliated Hospital of Air Force Medical University (No.2019JSYJ008).

Data Availability of Statement

The raw sequence data of transcriptome sequencing were deposited in the Sequence Read Archive (SRA) at NCBI under Bioproject PRJNA997380 (SUB13692495, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA997380). The original contributions presented in the study are included in the article and Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics Statement

The studies involving human participants were approved by the Ethics Committee of Tangdu Hospital, Fourth Military Medical University (No.202303-154). The specimens used in this study were not individually identifiable or traceable, and the waiver of informed consent would not have a negative impact on the rights and interests of the subjects. The informed consent waiver was approved by the Ethics Committee of Tangdu Hospital, Fourth Military Medical University, in accordance with the (Approaches to ethical review of biomedical research involving humans) by the State Health and Family Planning Commission, China. The animal study was approved by the Air Force Medical University's Institutional Animal Care and Use Committee (No.20230837).

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This research was funded by the Science and Technology Innovation Development Fund of the Second Affiliated Hospital of Air Force Medical University (No.2019JSYJ008).

Authors' Contributions

X-n W and R-cL designed this work; X-n W, R-c L, and R-x H performed the cell experiments and analyzed the data; X-n W, C L, and Z-w G performed the animal experiments; X-n W and X W prepared the original draft; K D supervised this work. All authors contributed to the article and approved the submitted version.

Conflicts of Interest

The authors declare no competing interests.

Declaration

We have not used any AI tools or technologies to prepare this manuscript.

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