

The Regulation of Macrophage Polarization by Hypoxia-PADI4 Coordination in Rheumatoid Arthritis

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Abstract

Background: Hypoxia, a common feature of rheumatoid arthritis (RA), induces the overexpression of peptidyl arginine deiminase 4 (PADI4) in fibroblast-like synoviocytes (FLSs) and macrophages. However, the roles of PADI4 and its inducer hypoxia in the regulation of macrophage polarization remain unclear. This study aimed to investigate the role of hypoxia-PADI4 for macrophage polarization in RA patients.

Methods: synovial tissue(ST) and synovial fluid(SF) were collected from 3 OA patients and 6 RA patients. The distribution of M1 and M2 in ST and cytokines in SF were examined by immunohistochemical analysis and BioPlex immunoassays. THP-1 macrophages and BMDM polarization were determined under normoxic (21% oxygen) or hypoxic (3% oxygen) conditions. The effects of PADI4 on macrophages were determined by transfection of adenovirus vector-coated PADI4 (AdPADI4) and the use of PADI4 inhibitor. To mimic the environment of RA joints, THP-1 macrophages polarization was examined after coculturing with RA-FLSs. Finally, the roles of PADI4 in joint synovial lesions on macrophage polarization were investigated in collagen-induced arthritis(CIA) rats.

Results: We found increased macrophage polarization of M1 and M2 in the RA ST, compared with OA ST. The ratio of M1/M2 for RA and OA was 1.633 ± 0.1443 and 2.544 ± 0.4429 , respectively. The concentration of M1- and M2-type cytokines was higher in RA than that in OA patients. Hypoxia contributed to the increase of the gene and protein expression of M1 and M2 markers. M1- but not M2-type gene expression showed a positive relationship with PADI4 expression while the level of expression of M2-type genes showed no significant difference. RA-FLSs could promote the copolarization of M1 and M2, and PADI4 inhibitor reversed M1 activation. The degree of joint swelling and destruction was effectively alleviated, and the number of macrophages especially M1 decreased in CIA rats after downregulating PADI4 expression.

Conclusion: Hypoxia is responsible for the copolarization of M1 and M2. Hypoxia-associated PADI4 is responsible for M1 macrophage activation, implying that inflammatory environment can be eased by decreasing PADI4 expression and improving the hypoxic environment.

Background

Rheumatoid arthritis (RA) is a common chronic autoimmune disease characterized by abnormal activation of the immune system, resulting in synovitis and destruction of joint function (1). Synovitis is characterized by an overabundance of RA fibroblast-like synoviocytes (RA-FLSs), monocytes, macrophages, and other immune cells within the RA synovial tissue (2). Among these cells, it has been well recognized that macrophages play an essential role in the initiation and perpetuation of RA by secreting multiple inflammatory and anti-inflammatory factors (3). Mature macrophages have two polarization states: classical activation (M1) and the alternative activation (M2) phenotype (4). M1 macrophages, induced by Th-1 cytokine interferon- γ (IFN- γ) or lipopolysaccharide (LPS), contribute to the development of inflammation and joint destruction by producing numerous pro-inflammatory factors,

such as IL-12 and TNF- α , and specifically expressing NOS-2 and CCR7 as biomarkers. The Th-2 cytokines IL-4, IL-10, and IL-13, however, cause macrophages to polarize into the M2 phenotype. They can then secrete anti-inflammatory cytokine IL-10, increasing the specific expression of the mannose receptor (CD206) (5). The high concentration of TNF- α in the RA joint synovium can promote M1 polarization (6), and the fibroblast factor secreted by RA-FLSs can promote M2 polarization (7). However, the role and molecular mechanism of M1/M2 in RA synovial lesions remain largely unknown.

An early step in the development of RA is the activation of RA-FLSs. This is followed by mass production of inflammatory mediators, including cytokines and chemokines, which mediate the recruitment and interaction with immune cells (8). This process enhances the oxygen consumption caused by the proliferation of cells in RA synovium, which leads to the oxygen tension in RA, indicated by the 2–4% values, which in diseased synovial tissues can be even lower than 1% of O₂ (hypoxia condition); healthy controls show levels around 8% (9). Macrophages, as well as other leukocytes, are sensitive to tissue hypoxia, which can increase inflammatory cytokine production (10), yet how hypoxia affects macrophage polarization is not well understood *in vitro*.

Peptidyl arginine deiminase IV (PADI4) is involved in the post-translational modification of arginine residues (11). A study revealed that a large number of activated macrophages and FLSs in RA synovial tissues are involved in the overexpression of PADI4 (12). The contribution of PADI4 to the apoptosis of RA-FLSs has been revealed (13). Considering the multiple roles of PADI4 in the regulation of gene expression and immunological functions, PADI4 may be a potential target for the therapy of autoimmune diseases. However, the roles of PADI4 and its inducer hypoxia in the regulation of macrophage polarization remain unclear.

This study aimed to investigate the polarization of macrophages in RA synovial tissue and elucidate the role of hypoxia-PADI4 in macrophage polarization. A collagen-induced arthritis (CIA) mouse model (13) was used to explore the PADI4-mediated development of inflammation.

Materials And Methods

Patients and controls

Clinical samples were the resident synovial tissue collected from patients undergoing knee arthroscopic or routine examination at Shanghai East Hospital, including six patients with RA and three patients with osteoarthritis (OA). All of the patients provided consent and met the diagnostic criteria of the American College of Rheumatology (ACR) criteria for RA (14) and OA (15). The Ethics Committee approved the study protocol of Shanghai East Hospital.

Animals

Twelve SD rats were administered intradermally at the base of the tail with a dose of bovine type II collagen (100 µg) emulsified in complete Freund's adjuvant on day 0. Then a booster injection was provided on day 21 with bovine type II collagen (100 µg) emulsified with incomplete Freund's adjuvant. The rats were then randomly divided into two groups: the CIA model group and the PADI4 inhibition group. Normal non-immunized rats were selected as the normal control group. The rats were sacrificed on the final day; joints were collected from all of the groups.

Immunohistochemical and immunofluorescence analyses

Synovial tissues from humans and joints from rats were fixed in 10% neutral buffered formalin and then embedded in paraffin. Paraffin sections were deparaffinized and rehydrated. After blocking with 3% H₂O₂, the sections were incubated with a goat polyclonal antibody against human CCR7 (1:500, Abcam, Cambridge, MA, US) and rabbit polyclonal antibody against human mannose receptor (CD206, 1:500, Abcam, Cambridge, MA, US) at 4°C overnight. Next, the sections were incubated with secondary antibody for 30 min at room temperature. Immunoreactive signals were visualized using DAB (diaminobenzidine 3). For double immunofluorescence staining, the sections were incubated with anti-CCR7 antibody or anti-CD206 antibody at 4°C overnight, then incubated with Donkey anti-Goat IgG Alexa Fluor 488 (1:200, Thermo Fisher, US) and Donkey anti-Rabbit IgG Alexa Fluor 594 (1:200, Thermo Fisher, US) for 30 min at room temperature. The cells were counterstained with DAPI (4',6-diamidino-2-phenylindole) and visualized under a fluorescence microscope.

Cytokine immunoassay

Synovial fluid samples were collected, three RA, and three OA samples as controls. Undiluted media samples were plated, and analytes (IL-1, IL-6, IL-8, IL-10, IL-12 IL-13, IL-17, IFN-γ, and TNF-α) were assessed according to the protocol included with the ProcartaPlex™ Platinum Human Multiplex Assay. Each plate was read on the BioPlex 200 (BioRad).

Cell culture

Human monocytic THP-1 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Roswell Park Memorial Institute medium (RPMI) 1640 culture medium containing 10% heat-inactivated fetal bovine serum (FBS), 20 mg/mL penicillin, and 20 mg/mL streptomycin. Cells were treated with 100 nM PMA (Sigma-Aldrich, #P8195) for 24 h and differentiated into M0 macrophages as previously reported (16), then the cells were further cultured in a normoxic (21% O₂) and a hypoxia (3% O₂) incubator. M1 was obtained from M0 by being treated with 20 µg/mL IFN-γ (PeproTech) and 100 ng/mL LPS (#L8630, Sigma), while M2 was treated with 20 µg/mL IL-4 (PeproTech) for another 48 h.

Bone marrow (BM) cells were harvested from the femurs and tibias of 6- to 10-week-old C57BL/6 mice (SJA Laboratory Animal Co., Ltd). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS (Wisent Biomart) and recombinant mouse M-CSF (40 ng/ml; PeproTech). After one week, BMDMs were replated and untreated (M0) macrophages were then stimulated with *Escherichia coli* LPS O111: B4 (100 ng/ml; Sigma) and IFN- γ (20 ng/ml; PeproTech) for 24 h (M1) or with IL-4 (20 ng/ml; PeproTech) for 24 h (M2).

Primary RA-FLSs were isolated from RA synovial tissues. After isolation from the tissue, RA-FLSs were grown further over four to six passages for our experiments. Then these cells were cocultured with THP-1 macrophages through a Transwell coculture system.

Virus vector infection

Adenovirus vectors expressing PADI4 (Genechem, China) were used in accordance with the manufacturer's instructions to transfect genes into M0. RT-PCR and Western blot were used to assess PADI4 expression.

Cocultures of RA-FLSs and M0

The coculture systems were established through a 24-well plate with an 8.0- μ m Pore Polycarbonate Membrane Insert (Corning, NY, US). To study the impact of the coculture system on macrophages, macrophages cocultured with RA-FLSs were treated as coculture groups, and the macrophages cocultured macrophages as control groups. M0 were seeded in 5×10^4 cells/well, and 1×10^4 cells/well RA-FLSs were seeded in the transwell inserts in another separated well. After 6 h of incubation, cells had become firmly attached to the wall. Then, the transwell inserts with RA-FLSs were moved to the wells containing the M0 macrophage and after that cocultured for 48–72 h. The coculture system was subjected to normoxia (21% O₂) and used as controls, and another set was placed under hypoxia conditions (3% O₂) in the incubator.

Quantitative real-time polymerase chain reaction

Total RNA from RA-FLSs and macrophages was extracted using TRIzol™ (Takara), and reverse transcribed using the Reverse Transcription System kit (Promega Corporation, US) according to the manufacturer's instructions. A quantitative real-time polymerase chain reaction (q-PCR) was performed using Premix Ex Taq SYBR Green PCR (Takara) on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA, US) according to the manufacturer's instructions. The primers used to amplify target gene products were as follows: human GAPDH 5'-ACCATGGGGAAGGTGAAG-3', 5'-AATGAAGGGGTCATTGATGG-3'; human PADI4 5'-TTCTCTAAGGCGGAAGCTTTT-3', 5'-AGCAGGGAACACACCTTCTC-3'; human CCR7 5'-GCATTTGTAGTCCTGCTGCG-3', 5'-CTCCTCAGATCTCACCCGTTG-3'; human TNF- α 5'-

TCTTCTCGAACCCCGAGTGAC-3', 5'-GGTACAGGCCCTCTGATG-3'; IL-10 5'-CACTGCTCTGTTGCCTGGTC-3', 5'-GAAGCATGTTAGGCAGGTTGC-3'; human NOS-2 5'-CATGAGCCCCTTCATCAATGC-3', 5'-TTGAAGTCTGTGTCCGAAGGC-3'; human TGF- β 5'-CCGAGAAGCGGTACCTGAAC-3', 5'-CGCCAGGAATTGTTGCTGTA-3'. murine GAPDH 5'-AGGTCGGTGTGAACGGATTTG-3', 5'-TG TAGACCATGTAGTTGAGGTCA-3'; murine PADI4 5'-TACCTGATGCTCCCATCCCA-3', 5'-TCCTAGGTACACCCAGAGGC-3'; murine CCL2 5'-CAGCCAGATGCAGTTAACGCCCCA-3', 5'-TGGGGTCAGCACAGACCTCTCTC-3'; murine TNF- α 5'-CCCTCACACTCAGATCATCTTCT-3', 5'-GCTACGACGTGGGCTACAG-3'; murine CCR7 5'-CGTGGTATTCTCGCCGATGT-3', 5'-TCTGCAAGAGAACAGAAGCCC-3'; murine IL-10 5'-GCAAGGGTGTCTCCTTCCTC-3', 5'-CTTGTTACTCGCCCCCTT-3'; murine Arg-1 5'-CTCCAAGCCAAAGTCCTTAGAG-3', 5'-AGGAGCTGTCATTAGGGACATC-3'. The relative expression of each gene was determined by Ct value ($\Delta\text{Ct} = \text{Ct target} - \text{Ct GAPDH}$), and the $\Delta\Delta\text{Ct}$ method ($\Delta\Delta\text{Ct} = \Delta\text{Ct sample} - \Delta\text{Ct control}$), which was used in the comparison with the control group.

Statistical analysis

Statistical analysis was performed using Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA, US). Data were collected from each experiment and compared between groups using analysis of variance (ANOVA) or t-tests (two-tailed distribution) as appropriate. A *P*-value of ≤ 0.05 was considered to be significant.

Results

M1 and M2 are both abundant in RA synovial tissues

To determine the distribution of M1 and M2 macrophages in synovial tissues from RA and OA, we performed immunostaining and double immunofluorescent staining. We found CCR7⁺ (M1 marker) and CD206⁺ (M2 marker) cells in the synovial tissues of both RA and OA (Fig. 1A). In RA, the mean ratio of M1/M2 was 1.633 ± 0.1443 , and in OA was 2.544 ± 0.4429 , i.e., the predominant form were the M1 macrophages (Fig. 1B) ($P < 0.05$). The results of double immunofluorescent staining showed the distribution of M1 and M2 in the RA group to be both more abundant than in the OA group, and the average fluorescence intensity of M2 was higher in RA than in OA (Fig.1 C&D) ($P < 0.05$).

BioPlex array was used to assess the expression of cytokines in synovial fluid. The Bioplex suspension array system allowed the simultaneous measurement of 10 human cytokines, including interleukins, interferons, and tumor necrosis factors. The results showed that the levels of M1 and M2 cytokines, IL-1, IL-4, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, IFN- γ , VEGF, and EGF to be significantly higher in RA than in OA (Fig. 1E) ($P < 0.05$), suggesting that the degree of inflammatory cell infiltration (both M1 and M2) in RA was more pronounced than in OA.

The expression of M1- and M2-related genes increases under hypoxia

As shown in Fig. 2A, qPCR revealed that gene expression of M1 markers (*Ccr7*, *Ccl2*, and *Il-1β*) and M2 markers (*Tgfbβ*, *Il-10*, and *Ccl18*) in PMA-treated THP-1 macrophages were significantly increased under 3% O₂ conditions. We then polarized M1 and M2 with IFN-γ, LPS, and IL-4. The results showed an elevation of hypoxia-induced M1 gene expression in M1, and M2 caused the same phenomenon, but the range of change was not as pronounced as with M1. Immunofluorescence assay was used to analyze the differential expression of M1 (CCR7) and M2 (CD206) in the 3% O₂ groups and 21% O₂ control, and the results were consistent with mRNA expression (Fig. 2B). Fig.2C showed that the mRNA levels of *Ccl2* and TNF-α increased under 3% O₂ in BM M0 and M1, the change of M2 marker (Arg-1) was consistent with THP1-derived macrophages. These observations suggest that hypoxia can increase the level of expression of genes related to M1 and M2 genes.

Increased PADI4 expression induced by hypoxia affects the polarization of macrophages

To investigate the effect of hypoxia on PADI4 expression, THP-1 macrophages were cultured under conditions of different oxygen concentrations (3% O₂ and 21% O₂). After culture for 36–48 h, the gene expression of PADI4 in macrophages was determined using qPCR and Western blotting. As shown in Fig. 3A, mRNA, and protein levels of PADI4 were upregulated in cells cultured under 3% O₂ conditions relative to those cultured with 21% O₂. We then evaluated the effect of PADI4 on the polarization of macrophages. Cells were incubated in a hypoxic environment in the presence of Cl-amidine, the PADI4 inhibitor, to block PADI4 (Fig. 3A). As shown in Fig. 3B, the mRNA levels of M1 markers decreased, while the mRNA levels of most M2 markers did not change significantly in THP1-derived macrophages. In contrast, the expression of M2 markers (IL-10, Arg-1) increased in BMDM. These results may indicate that PADI4 affects the polarization of macrophages, especially on M1 under hypoxic conditions.

PADI4-induced macrophages convert to M1

To determine whether PADI4 could affect the polarization process of macrophages, we used adenovirus vectors to determine PADI4 overexpression macrophages in normoxia. THP-1-derived macrophages were infected with AdPADI4 and AdGFP as controls. The infection effect was assessed using qPCR and Western blot (Fig. 4A). As shown in Fig. 4B, significant increases were found in the mRNA expression of M1 genes in the *Padi4* overexpression group compared with the control group under normoxic conditions ($P < 0.05$). There were no readily visible differences in M2 genes between these two groups. BMDM showed an increase of M1 marker expression and a decrease in M2 markers expression. This phenomenon also happened in M1, which was further polarized into using IFN-γ and LPS (Fig. 4D). The

results indicate that PADI4 may be associated with the process by which macrophages convert to the M1 phenotype, but it was not found to have any effect on M2 polarization. The increases in the expression of M2-related genes may have been due to hypoxia.

RA-FLSs-induced PADI4 overexpression affects M1 gene expression

The effects of coculturing with RA-FLSs on macrophage polarization are shown in Figure 6. For macrophages, the quantitative RT-PCR results showed that the mRNA levels of M1 and M2 genes were higher in the coculture groups than in the control groups, which had significant differences (Fig. 5A) ($P < 0.05$). The addition of the Cl-amidine into the coculture system resulted in a decrease in the expression of M1 inflammatory genes in macrophages and an increase in M2 inflammatory genes (Fig. 5B) ($P < 0.05$). These results suggested that reducing *Padi4* down-regulated M1 activation and hypoxia and enhanced the M2 polarization in this coculture system.

Macrophages infiltration in the synovial tissue is decreased by *Padi4* downregulation in CIA rats

After the booster immunization, we evaluated the arthritic scores every 7 days for 63 days. The symptoms associated with arthritis gradually appeared in all of the CIA rats on the 7th day and continued to develop severe arthritis until they reached a plateau between days 28 and 35. Si-*Padi4*-MSN was injected on the 35th day. The control group treated with MSN showed severe swelling, erythema, and joint rigidity in the paw, while the administration of si-*Padi4*-MSN significantly lowered the arthritic scores. Immunohistochemical analysis of the synovial tissue revealed that *Padi4* downregulation rats exhibited reduced macrophages(CD68⁺) infiltration compared with the control group—mainly composed by M1(CCR7⁺). The general photos and X-ray pictures and similar results are shown in Fig.6. Collectively, our results suggested that PADI4 exhibited a certain effect on the CIA.

Discussion

Macrophages, the main resource of TNF- α and other pathogenic inflammatory factors, play a crucial role in the inflammatory response. Previous work has shown that the number of macrophages correlated with the degree of synovial hyperplasia and is positively correlated with the patient's DAS28 score and the degree of joint bone erosion (17). Several studies have shown that M1 dominates the inflammatory environment in the RA joint, in which synovial fluid has an M1/M2 ratio of 32.76 ± 11.02 (18). Using patient synovia tissue, we confirmed that both M1 and M2 were facilitated in the RA joint synovia tissue, with an M1/M2 ratio of 2.544 ± 0.4429 . The concentration of M1 and M2 relative cytokines were more abundant (TNF- α , IL-6, IL-1, IL-4, IL-8, IL-10, IL-12, IL-15, IL-17, IFN- γ , GM-CSF, and VEGF) compared to OA. A recent study reported that exposure of M2 macrophages to the particular combination of c-IgG and toll-

like receptor (TLR) ligands, as occurs in the synovia in most patients with RA, strongly promotes inflammation (19). Previous immunohistochemistry data showed that more than 80% of CD163⁺ macrophages (M2) were responsible for secreting TNF- α in synovia of patients with RA (20). Our observations provided experimental evidence that the polarization of M1 and M2 were indeed enhanced in RA synovia tissue, resulting the development of inflammation.

In previous work, we found that PADI4 is overexpressed in RA-FLSs and promotes the proliferation of RA-FLSs under hypoxic conditions (13). It has been reported PADI4 expression in CD68 macrophages, and it showed a positive correlation with the expression of several genes associated with macrophage activation and function (21). However, the effect of PADI4 on macrophage polarization requires further investigation. In this study, we confirmed a marked overexpression of PADI4 expression in macrophages under hypoxic conditions. Here, we substantiate the role of PADI4 by showing that the PADI4 knockdown indeed weakened M1 marker expression and its overexpression-enhanced M1 polarization, whereas the M2 marker expression was unaffected (Fig. 4). Based on these findings, we suggest that hypoxia enhances M1 and M2 polarization, and hypoxia-induced PADI4 mainly regulates M1 activation.

Various proinflammatory factors secreted from FLSs can influence the interaction of FLS and other immune cells, especially macrophages, including activating M1 polarization and anti-inflammatory effects. Coculture models of RA-FLSs and THP-1 macrophages *in vitro* resulted in the activation of M1 and M2, and *Padi4* expression increased in macrophages. In the presence of the PADI4 inhibitor Cl-amidine, M1-related genes were inhibited, especially *Tnf- α* , and the expression of M2-related genes increased (Fig. 5). These results suggested that targeting PADI4 helped to suppress the pro-inflammatory process. Further investigation is needed to determine whether PADI4 contributes to pro-inflammation in the mouse model.

Si-RNA-MSN, a nanoparticle carrier we have used in previous studies that is slowly released and can easily reach an effective concentration in the body, was injected into CIA rats to knockdown *Padi4*. Our study demonstrated that joint swelling and macrophages infiltration were improved after PADI4 inhibition, especially the number of M1. M2 marker did not perform well due to the difference in the phenotype of human and mice, however, the significant decrease in the number of M1 can also prove our conclusion. Citrullination, catalyzed by PADI4, is implicated in the host cell apoptosis and immune cell clearance post-infection (22). It has been reported that citrullination may dampen host defense and promote pathogen survival (23). Although PADI4 is well-known to be involved in RA, especially in cell apoptosis, our research gives evidence that targeting PADI4 is promising to weaken the active immune cell infiltration and alleviate chronic inflammation.

Conclusion

All of the data in our paper demonstrated that The PADI4 inhibitor could alleviate the symptoms by reducing polarization of M1 then affecting the M1/M2 ratio and their infiltration degree. The underlying

mechanism may be the regulation of the hypoxia-PADI4 axis and PADI4 expression level is a critical factor for treating RA.

Abbreviations

Adenovirus: Ad

Arg-1: arginase 1

CCR7: C-C chemokine receptor type 7

CD206: Mannose Receptor

DAB: diaminobezidin

FBS: fetal bovine serum

FLS: fibroblast-like synoviocytes

GFP: green fluorescent protein

HIF: hypoxia-inducible factor

IF: Immunofluorescence

IgG immunoglobulin G

IHC: Immunohistochemistry

IL-1 β : interleukin-1 β

HRP: Horseradish Peroxidase

OA: Osteoarthritis

PADI: Peptidylarginine deiminases

PMA Phorbol-12-myristate-13-acetate

PS: Penicillin & Streptomycin

TGF- β : transforming growth factor- β

TNF- α : tumor necrosis factor- α

IL-6: interleukin-6

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Tong Ji University (DFSC-2019(CR)-03). Informed consent was acquired from all the participants in the study.

Consent for publication

All authors agreed the publication of final manuscript in this journal.

Availability of data and materials

Data are available from the authors in the current study no matter when it has reasonable request.

Competing interests

There are no competing interests.

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Authors' contributions

YC, DML, MZ, and LF participated in designing the whole study and progression. YS and LW were involved in data collection and analysis. YS, YD and LL gave support to software analysis and verification. YC, DML, MZ, and LF wrote and modified the manuscript and prepared figures. Everyone agrees to this division during the current study.

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Figures

Figure 1

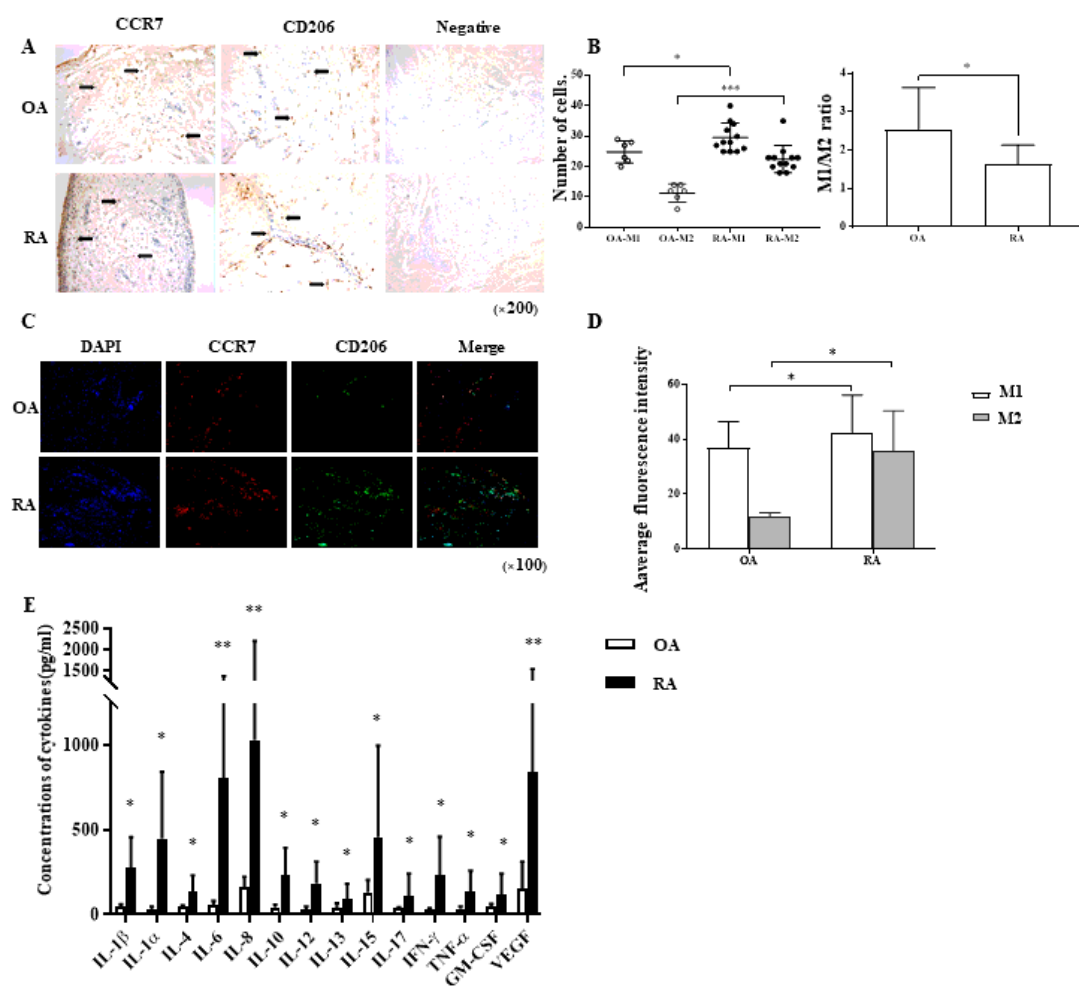


Figure 1

The distribution of M1 and M2 macrophages in arthritic synovial tissues from RA and patients with OA. (A) Immunohistochemistry staining. Continuous sections of RA or OA synovial tissues were probed with anti-CCR7 Ab and anti-CD206 Ab. Arrows indicate positive cells. Original magnification: x200. (B) Comparison of the number and ratio of M1(CCR7) and M2(CD206) macrophages in RA (n = 6) and OA (n = 3). (C) Double immunofluorescent staining. The sections of RA or OA synovial tissues were probed with

anti-CCR7 Ab and anti-CD206 Ab, followed by incubation with the second antibody the anti-goat IgG Alexa Fluor 488 (green) or anti-mouse IgG Alexa Fluor 594 (red). Original magnification: x100. (D) Fluorescence intensity of M1 and M2 in RA (n = 3) and OA (n = 3). (E) Level of IL-1, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IFN- γ , and TNF- α in synovial fluid isolated from RA and OA was assayed by the Bioplex assay. RA (n = 6) and OA (n = 3); anti-CD206 Ab, Mannose Receptor antibody; IgG, immunoglobulin G; OA, osteoarthritis; RA < 0.05.

Figure 2

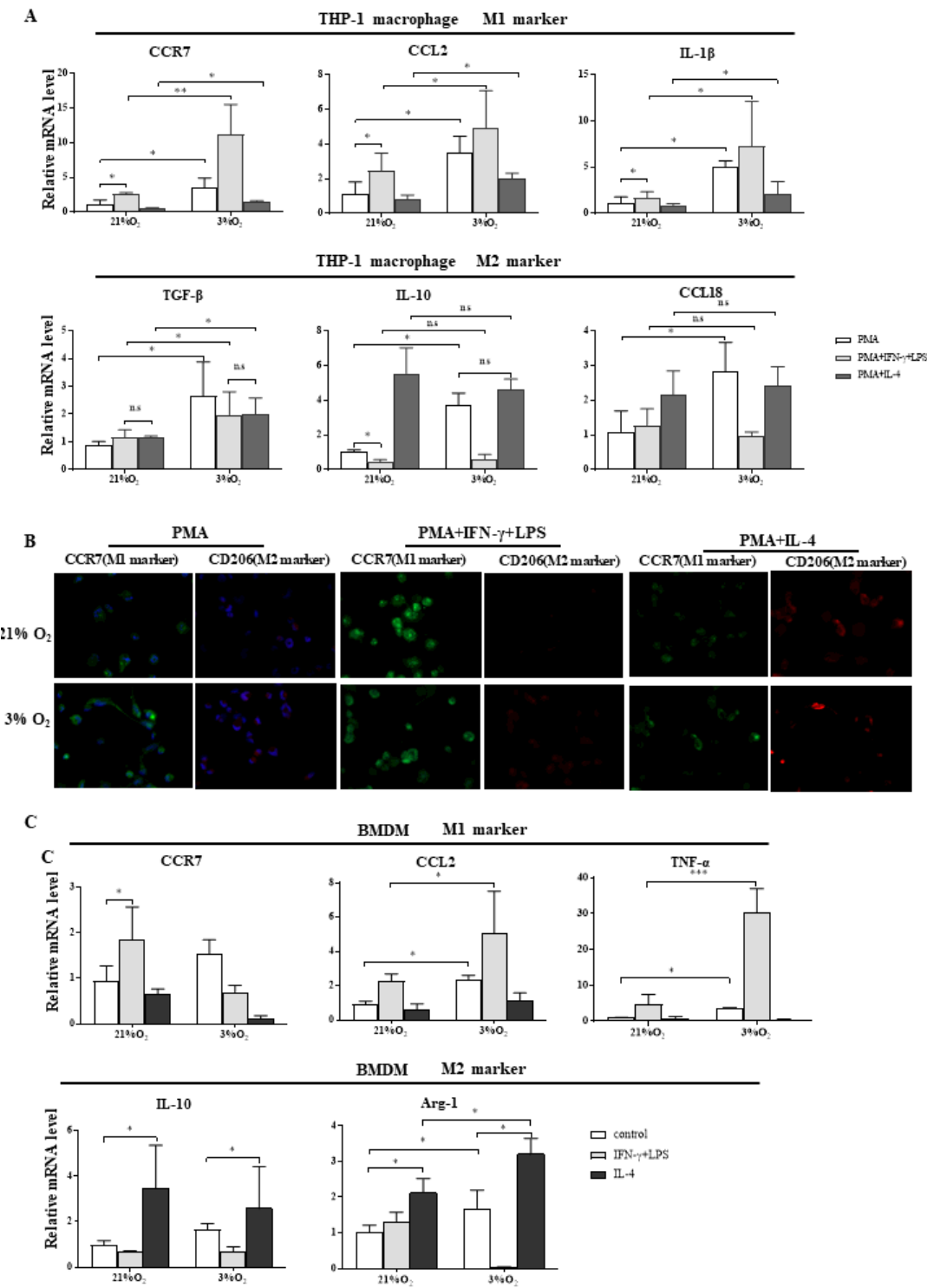


Figure 2

Hypoxia affects the expression of polarization-related genes by macrophages. (A) THP-1 cells were treated with PMA, and 24 h later were incubated at 21%O₂ and 3% O₂. Cells were polarized into M1 or M2 with IFN- γ and LPS or IL-4 for another 24 h. The expression of Ccr7, Ccl2, Il-1 β , Ccl18, Tgf β , and Il-10 mRNA in the cells was detected using qualitative PCR. (B) Immunofluorescence for the polarization of THP1-1-derived macrophages. M1 and M2 were probed with anti-CD197 Ab and anti-CD206 Ab, followed by incubation with second antibody anti-goat IgG Alexa Fluor 488 (green) or anti-mouse IgG Alexa Fluor 594 (red). Original magnification: $\times 400$. Green fluorescence indicates M1. Red fluorescence indicates M2. The nuclei are stained blue with DAPI. (C) The same experiment was performed on bone marrow-derived macrophages (BMDM). Data are representative of three independent experiments. Bars indicate mean \pm standard error of the mean (SEM), Unpaired t-test, * P < 0.05; ** P < 0.01; *** P < 0.001, anti-CD197 Ab, CCR7 antibody; anti-CD206 Ab, mannose receptor antibody; IgG, immunoglobulin G;

Figure 3

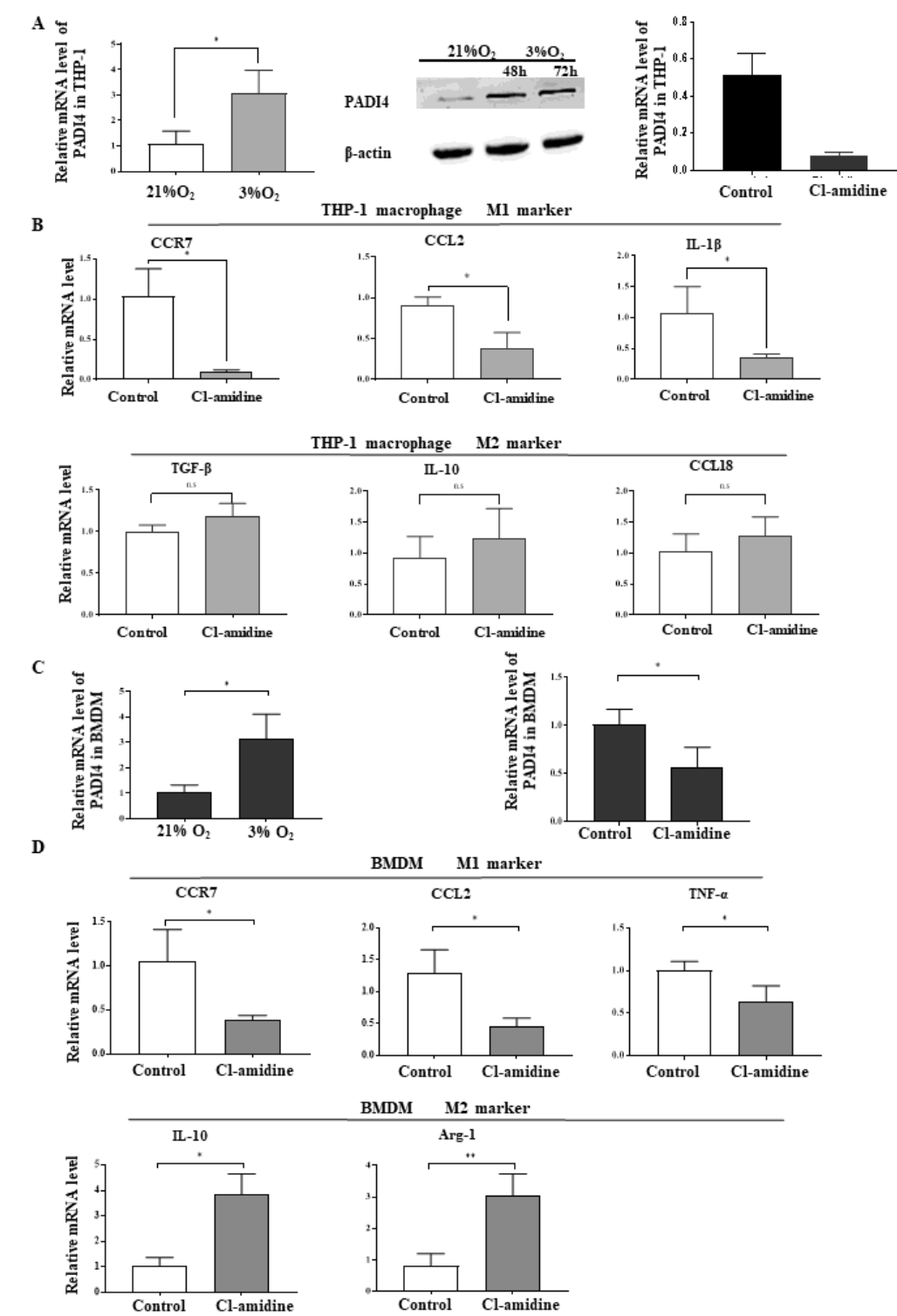


Figure 3

Expression of Padi4 and polarization of macrophages after the introduction of PADI4 inhibitor in hypoxia. (A) THP-1 cells were treated with PMA. After 24 h, they were incubated at 21% O₂ and 3% O₂. PADI4 mRNA and protein expression levels were determined using RT-PCR. Then, they were incubated in a medium containing Cl-amidine at 3% O₂ for downregulating the expression of PADI4. The expression of Padi4 was determined using quantitative RT-PCR. (B) M1 markers (CCR7, CCL2, TNF-α), M2 markers (IL-

10, CCL18, TGF β) mRNA in THP-1 macrophages were determined by quantitative RT-PCR after using PADI4 inhibitor. (C) The expression of PADI4 in BMDMs was tested after treating with hypoxia and PADI4 inhibitor. (D) M1 and M2 markers were tested in BMDM. Bars indicate mean \pm SEM. A Student's t-test was performed using the P-value indicated * $P < 0.05$, ** $P < 0.01$, "n.s." indicates "not-significant." PMA, Phorbol 12-myristate 13-acetate; PADI4, peptidyl arginine deiminase type 4; RT-PCR, reverse transcriptase-polymerase chain reaction.

Figure 4

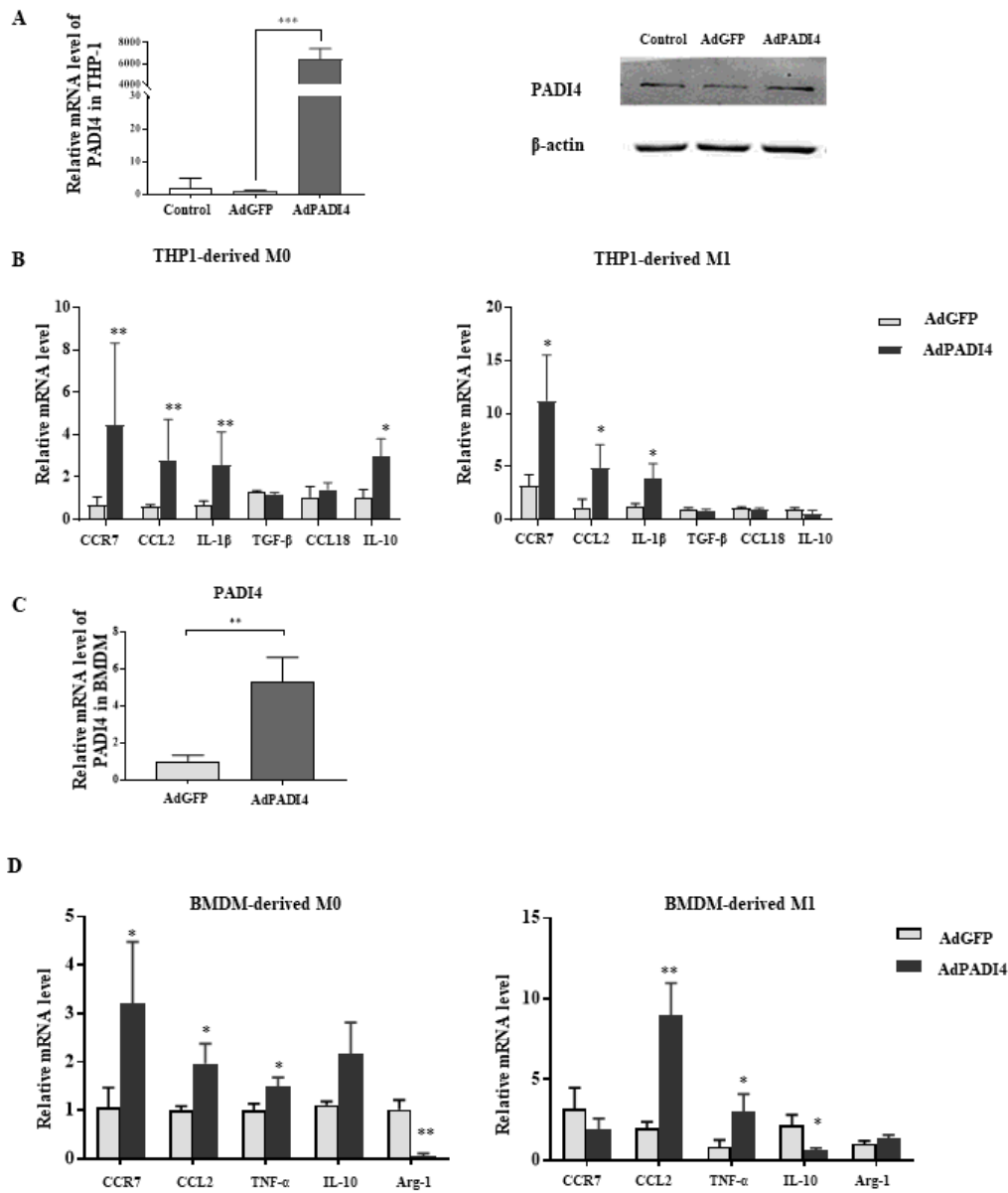


Figure 4

Overexpression of PADI4 and its effect on the polarization of macrophages under normoxia. (A) THP-1-derived macrophages were infected with AdPADI4 and incubated at 21% O₂. PADI4 mRNA and protein levels were determined using RT-PCR and Western blot after 48 h. (B) M1 marker (Ccr7, Ccl2, Il-1 β) and M2 marker (Tgf- β , Il-10, Ccl18) mRNA were determined in THP1-derived M0 and M1 using quantitative RT-PCR. (C) The expression of PADI4 was upregulated in BMDM using AdPADI4. (D) The mRNA levels of M1 and M2 markers in BM-derived M0 and M1 were determined. Data are representative of three independent experiments. "n.s." indicates "not-significant" in student t-test. "n.s." indicates "not-significant," ** P<0.01, *** P<0.001. PADI4, peptidyl arginine deiminase type 4; RT-PCR, reverse transcriptase-polymerase chain reaction.

Figure 5

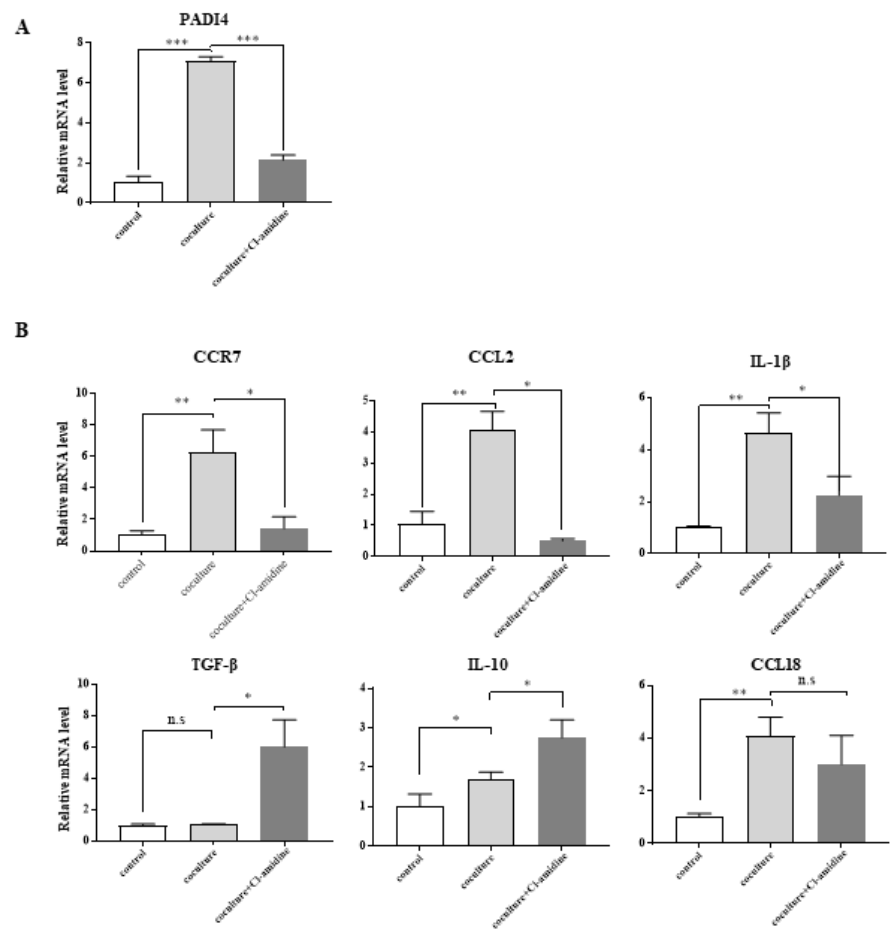


Figure 5

Effects of PADI4 on the expression of genes related to inflammation by microculture coculture system. (A) The expression of Padi4, Tnf- α , Ccr7, Nos-2, Il-10, Ccl18, and cocultured and control THP-1 macrophages was determined using quantitative RT-PCR. (B) Effects of PADI4 inhibitor on the expression of genes related to inflammation by macrophages. Macrophages in the control group and coculture group were treated with CI-amidine and then incubated at 3% O₂ for 24 h. The expression of TNF- α , Ccr7,

Arg-1, Il-10, Ccl18, and Tgfβ mRNA was determined using quantitative RT-PCR. Bars indicate mean ± SEM, a Student-test, "n.s." indicates "not-significant," * P < 0.05, ** P < 0.01, *** P < 0.001.

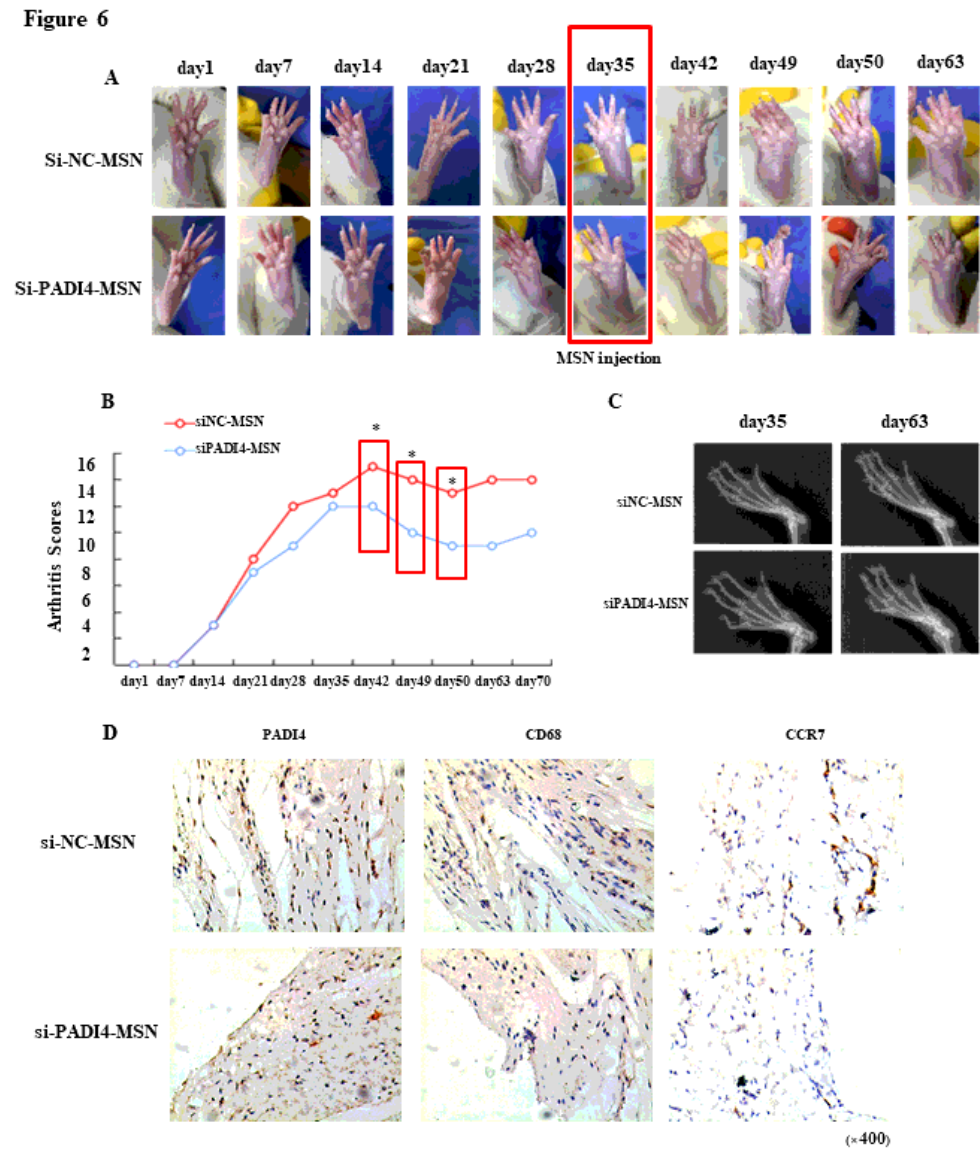


Figure 6

Padi4 knockdown significantly decreased joints damage in CIA rats. (A) Joints swelling was observed during booster immunization, MSN was injected on day 35. (B) Mean arthritic scores evaluated the severity of arthritis. (C) X-ray pictures of rat hind paws in different groups. The X-ray was performed on

day 35 and 63. Treatment with Baicalin significantly decreased histological damage in joints of CIA rats. (D) The rats were sacrificed on day 63, and their joints were harvested. PADI4, macrophages marker (CD68) and M1 marker (CCR7) stained with immunohistochemistry. The data were analyzed using a Student's t-test. The error bars represent the SEM. * $P < 0.05$.