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Research article

Role of the *CXCR4-Gnαq-Plcβ* signaling pathway in the pathogenesis of collagen-induced arthritis in rats

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which immune cells and inflammatory cytokines are abnormally activated, leading to immunoregulatory dysfunction in the body and triggering systemic inflammatory responses. The interaction between CXC chemokine receptor 4 (CXCR4) and heterotrimeric G-protein α-subunit Gαq (Gnαq) activates phospholipase Cβ (PLCβ), which influences the expression of downstream effectors and participates widely in the onset and development of various diseases, thus suggesting the potential involvement of these molecules in RA pathogenesis. Therefore, the present study aimed to determine whether the *CXCR4-Gnαq-PLCβ* signaling pathway participates in the onset and development of RA. Using a collagen-induced arthritis (CIA) rat model, we found that compared with the control (healthy) rat group, CIA rats exhibited highly time-dependent arthritis, with the maximum arthritis score occurring in week 3. In contrast to the splenic and joint tissue of control rats, CIA rats showed obvious hyperplasia in the lymphoid white pulp and main germination centers of the spleen, narrowing of joint cavities, and inflammatory cellular infiltration on articular surfaces. The serum levels of expression of IL-1β, IL-4, IL-6, and TNF-α were significantly elevated ($P < 0.05$, $P < 0.01$). Core genes of the *CXCR4-Gnαq-PLCβ* pathway, namely *CXCR4*, *Gnαq*, *PLCβ1*, *MMP1*, and *MMP3*, also showed a significant increase in mRNA and protein expression levels ($P < 0.05$, $P < 0.01$). Proteins related to the CXCR4-Gnαq-PLCβ pathway were mainly localized to the red and white pulp regions in the spleen as well as in stromal, endothelial, and subdifferentiated synovial cells in the joints. These results indicated that CXCR4 is dependent on Gnαq for inducing the expression of PLCβ1 and stimulation of secretion of inflammatory cytokines by inflammatory cells. This consequently affects the expression of matrix metalloproteinases (MMPs), which serve as downstream effectors, thereby promoting RA pathogenesis. Our findings play an important role in elucidating the mechanisms of the onset and development of RA.

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1. Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease accompanied by systemic joint deformities. The disease is mainly characterized pathologically by invasive synovitis, while it can lead to progressive cartilage erosion, degeneration, and bone destruction during progression [1]. A wide variety of cells, including macrophages, T-cells, B-cells, dendritic cells, and synovial cells, is involved in the onset and disease course of RA. During RA development, these cells undergo abnormal proliferation and differentiation, further secreting increased amounts of proinflammatory cytokines. This leads to prolongation and persistence of the symptomatic period of synovitis, consequently resulting in severe clinical manifestations. Although the roles of many genes and mechanisms in RA development have been investigated, the exact mechanism of RA pathogenesis remains unclear [2,3]

CXC chemokine receptor 4 (CXCR4) is a chemokine receptor expressed in various cells of the immune and central nervous systems [4,5]. In particular, it is expressed on the surfaces of articular chondrocytes [6,7]. The activation of CXCR4 can induce the secretion of various inflammatory cytokines, causing chondrocyte apoptosis and damage [8,9]. The expression of CXCR4, which is possibly primarily associated with cell migration, angiogenesis, and inflammation, is upregulated in endothelial cells [10]. Surface molecule analysis of B-cells in the peripheral blood of many patients with RA has revealed that CXCR4 serves an important role in synovial membrane migration, proliferation, and cytokine production of B-cells [11]. Gαq, an α-subunit of Gq, is encoded by *Gnαq* and belongs to the Gq/11 subfamily of G proteins. It is widely expressed in mammalian cells and undergoes coupling with various receptors, thereby activating channel proteins, enzymes, and other effector molecules [12,13]. *Gnαq* is involved in immune regulation and autoimmune diseases, playing a key role in regulating dendritic cell migration, regulatory B-cell (Breg) differentiation, and T-cell survival. It also participates in the onset and progression of RA through the regulation of lymphocyte survival and death [14,15]. Further research has demonstrated that CXCR4 activates phosphatidylinositol-specific phospholipase C β1 (PLCβ1) and the expression of downstream effector proteins through members of the Gnαq family, thereby serving a crucial role in coordinating the functions of the immune system.

Despite confirmation by many studies that CXCR4 and Gnαq regulate the expression of multiple genes during the onset and development of RA, no direct data exist regarding the involvement of the *CXCR4-Gnαq-PLCβ* signaling pathway in RA onset and development. We hypothesized that CXCR4 may be dependent on Gnαq for activation of the expression of PLCβ1 and stimulation of inflammatory cytokine secretion by inflammatory cells. This may consequently affect the expression of matrix metalloproteinases (MMPs), which serve as downstream effectors, thereby promoting the pathogenesis of RA. Therefore, we established a collagen-induced arthritis (CIA) rat model to confirm our hypothesis. Our results may provide the basis for understanding the mechanisms of RA onset and development and achieving targeted clinical treatment of RA in the future.

2. Materials and methods

2.1. Experimental animal grouping and sample collection

For this study, 24 specific pathogen-free (SPF) healthy female Wistar rats aged 6–8 weeks with similar body weights (170 ± 10 g) (License No.: SCXK (Jing) 2019–0010, SpePharm (Beijing) Bio. Co., Ltd., Beijing, China) were reared at the Laboratory Animal Center of Shanxi University of Chinese Medicine (Jinzhong, PR China). Rats were randomly divided into 2 groups, the control (n = 12) and CIA model (n = 12) group, and allowed to acclimatize to their rearing environment for 1 week. Consequently, bovine type II collagen solution (Cat # 20022, Chondrex, Inc., Redmond, WA, USA) was mixed with Freund's Complete Adjuvant (Lot. #F5881, Sigma-Aldrich Pte Ltd, St. Louis, MO, USA) in a 1:1 ratio to form a white emulsion (1 mg/mL). Rats received subcutaneous injections of the emulsion on the back (four points) and at the base of the tail (one point), with 0.1 mL injected at each point. After 7 d, booster immunization was performed by intraperitoneal injection (0.3 mL/rat) of a mixture of equal parts of bovine type II collagen solution and Freund's Incomplete Adjuvant (Lot. #F5506, Sigma-Aldrich Pte Ltd). The arthritic conditions of rats in both groups were observed 14 d after the booster injection. Arthritis severity was assessed using the following scoring criteria: 0 = no arthritis, 1 = mild/moderate erythema and swelling, 2 = severe swelling of the entire paw, 3 = arthritis affecting three joints, 4 = arthritis affecting all joints, 5 = paw deformity/joint ankylosis. A score of >8 indicated successful construction of the CIA model.

Rats were provided with maintenance feed for rats and mice (Cat. # SPF-F02-001, SpePharm Bio. Co., Ltd., Beijing, China) daily and given ad libitum access to food and water. They were reared in an environment with a 12 h light/12 h dark photocycle, temperature of (23 ± 2) °C, and relative humidity of (50 ± 15) %. All animal experiments were approved by the Institutional Animal Ethical Committee of Shanxi University of Chinese Medicine (approval no. AWE202302079). After fasting for 12 h, all rats were euthanized by intraperitoneal injection of 1 % pentobarbital sodium (50 mg/kg body weight, Cat. # 11715, Sigma-Aldrich Pte Ltd, St. Louis, MO, USA). All animal tissue samples were collected after euthanasia. During the experiment every effort was made to minimize the pain distress and death of animals.

The total duration of the experiment was 6 weeks (prefeeding for 1 week followed by the actual experiment over 5 weeks). Rats of all groups were fasted overnight (12 h) upon completion of the experiment, and subsequently anesthetized with isoflurane on the following day. Blood was collected from the abdominal aorta of anesthetized rats, and serum was obtained by centrifuging the blood samples at 1500×*g* and 25 ± 2 °C for 10 min. After blood sample collection, rats were euthanized by cervical dislocation. The ankle joint of left hind limb and spleen (approximately 0.5 cm × 0.5 cm × 0.5 cm) were obtained from each rat and fixed in 4 % paraformaldehyde, while the right ankle joint was stored at −80 °C.

2.2. Joint scoring and body weight measurement

Clinical scoring was performed on all limbs of experimental rats once a week by two independent observers blinded to grouping. Scores were assigned for an integrated index that assessed both disease severity and the number of affected limbs. The body weight of rats was measured weekly, and all changes were statistically analyzed.

2.3. Pathological examination of splenic and joint tissues

The splenic and joint tissues of rats of were fixed in 4 % paraformaldehyde for at least 24 h. Joint tissues were decalcified in 10 % EDTA decalcifying solution for 5 weeks, cleared in xylene, embedded in paraffin, sectioned into 5 μm-thick slices, floated on a 37 °C warm water bath, baked in a 60 °C oven, deparaffinized in xylene, and dehydrated through a graded series of alcohol. Splenic tissues were stained with hematoxylin and eosin (H&E), whereas joint tissues were subjected to Masson's trichrome staining. Pathological changes in the spleen and joints were examined under an optical microscope (BX51, Olympus Co., Ltd., Tokyo, Japan).

2.4. Measurement of serum inflammatory cytokines

The serum levels of inflammatory cytokines, IL-1β, IL-4, IL-6, and TNF-α, were measured using an ELISA assay kit (Cat. # ml037373, ml102825, ml102828, and ml002859; Enzyme-linked Biotech. Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Standard, blank, and test sample wells were established in triplicate and sequentially subjected to dilution, enzyme addition, incubation, washing, color development, and reaction termination. Optical density (OD) values were measured at a wavelength of 450 nm, and the levels of inflammatory cytokines in samples were calculated using the standard curve.

2.5. Extraction of total RNA from splenic and joint tissues of rats

Frozen splenic and joint tissues were ground in liquid nitrogen. Total RNA was extracted using a total RNA extraction kit (Cat # 20211222, 20220415, and 20220620; Solarbio Sci. & Tech. Co., Ltd., Beijing, China) according to the manufacturer's instructions. The concentration and purity of extracted total RNA were quantified using a microvolume spectrophotometer (Nanodrop ND-2000, Nanodrop Tech., Wilmington, DE, USA). Subsequently, the extracted total RNA was stored at −80 °C. Reverse transcription was performed using a M5 Sprint qPCR RT kit with gDNA remover (Cat. # 22DB0705, Mei5 Biotech. Co., Ltd., Beijing, China) according to the manufacturer's instructions. Each 20 μL reaction system was set up as follows: (1) 2.0 μL of 5 × Sprint gDNA Remover Mix was added to 0.01–1 μg of total RNA, and the total volume was made up to 10 μL by adding double distilled H_2O (dd H_2O). The reaction mixture was incubated at 42 °C for 2 min and subsequently cooled on ice. (2) 4.0 μL of 5 × M5 Sprint RT Mix and 6 μL of DEPC-ddH_2O was added to the reaction system described in Step (1). The resultant mixture was incubated at 50 °C for 5 min and 85 °C for 5 s, and subsequently stored at −20 °C.

2.6. Primer design and synthesis

Based on the rat *CXCR*4, *Gnαq*, *PLCβ*1, *MMP*1, and *MMP*3 cDNA sequences deposited in the NCBI GenBank, five pairs of specific primers were designed in Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by TsingKe Bio. Tech. (Beijing, China) (Table 1). PCR amplification was performed using the following PCR reaction system (15 μL): 0.6 μL each of the forward and reverse primers (10 μmol L^{-1}), 0.8 μL of the cDNA template, 5.5 μL of ddH_2O, and 7.5 μL of 2 × M5 HiPer Taq HiFi PCR mix (with blue dye) (Cat. # MF002-05, Mei5 Biotech. Co., Ltd.). Reaction conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and then 72 °C for 5 min. PCR products were subjected to detection by electrophoresis in 2.0% agarose gel. Purification and extraction of the *CXCR*4, *Gnαq*, *PLCβ*1, *MMP*1, and *MMP*3 PCR amplification products were performed using a quick gel extraction kit (Cat #D1200, Solarbio Sci. & Tech. Co., Ltd.) in accordance with the manufacturer's instructions.

Table 1
Oligo-nucleotide primers pairs for genes related to *CXCR*4-*Gnαq*-*PLCβ* Signaling Pathway.

Genes	GenBank accession No.	Primer sequence (5′-3′)	Product/bp
*CXCR*4	NM_022205.3	Forward: CTGAACCCCATCCTCTACGC Reverse: GAAGAGTGTCCACCCCGTTT	128
Gnαq	NM_031036.1	Forward: AATGCTACGATAGACGGCGAG Reverse: ATAGGAAGGGTCAGCCACAC	89
*PLCβ*1	NM_001077641.2	Forward: TCCGAGGCTCCAAGTGAAAC Reverse: AGATCCTCTGTTTTGGCGGG	146
*MMP*1	NM_001134530.1	Forward: AACAGCAGAAGATTTCCCTGG Reverse: TCGGAGGCTAAATCTGCGTT	107
*MMP*3	NM_133523.3	Forward: TCCCCTGATGTCCTCGTGGT Reverse: GGGTCCTGAGAGATTTTCGC	160

2.7. Preparation of plasmid standards

Extracted DNA fragments were ligated to the *p*EASY®-T1 Cloning Vector using the *p*EASY®-T1 Cloning Kit (Cat. # CT101-01, TransGen Biotech. Co., Ltd., Beijing, China) according to the manufacturer's instructions. The ligation products were added to DH5α competent *Escherichia coli* cells (Cat. #C1100, Solarbio Sci. & Tech. Co., Ltd.) and cultured in an inverted position in Luria Broth (LB) solid medium containing ampicillin (AMP) at 37 °C for 12 h. Target single colonies were picked, inoculated in AMP-containing LB liquid medium, and cultured with shaking at 180 r/min and 37 °C for 12 h. Plasmid DNA was extracted using the GenElute HP Plasmid Maxiprep Kit (Cat. # NA0300-1 KT, Sigma-Aldrich Pte Ltd) according to the manufacturer's instructions. Plasmid copy number was calculated, and plasmid standards were established using six 10 × dilutions (10^3-10^8).

2.8. Measurement of mRNA levels of the CXCR4-Gnαq-PLCβ signaling pathway in splenic and joint tissues of rats

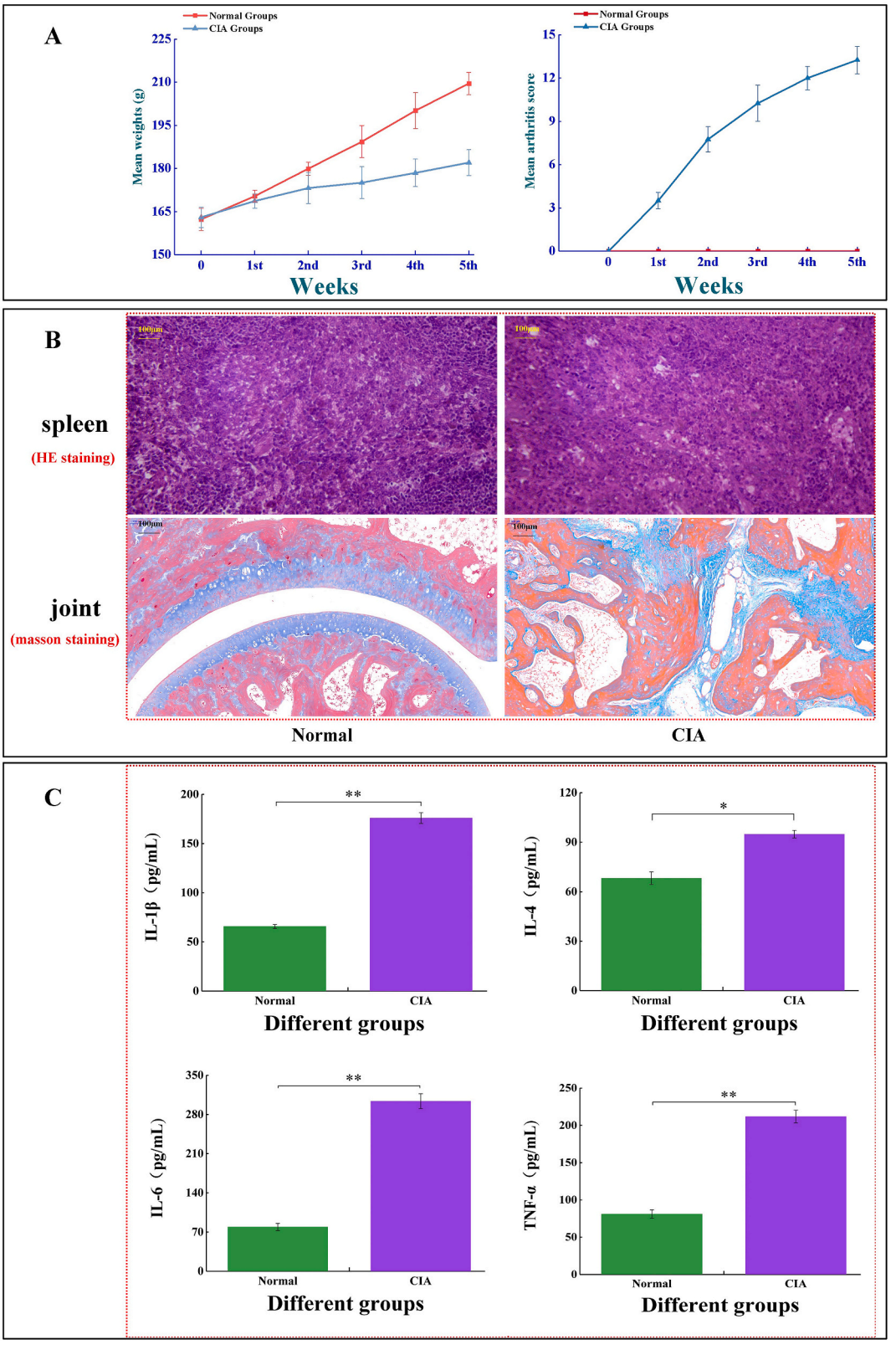
Plasmid standard numbers (copies/μL) for *CXCR4*, *Gnαq*, *PLCβ1*, *MMP1*, and *MMP3* in the qRT-PCR reaction system were separately calculated using the following formula: plasmid copy number (copies/μL) = 6.02×10^{23} (copies/mol) × plasmid concentration (μg/μL)/(DNA length × 660). The following reaction system (20 μL) was prepared according to the instructions provided with the TransStart® Top Green qPCR SuperMix kit (Cat. # AQ131, TransGen Biotech. Co., Ltd.): 0.5 μL each of the forward primer (10 μmol/L) and reverse primer (10 μmol/L), 2.0 μL of cDNA template, 10.0 μL of 2 × TransStart® Top Green qPCR SuperMix, and 7.0 μL of nuclease-free water. Six reactions were set up for each sample. Reaction steps were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 35 s, and then dissociation at 97 °C for 30 s, 65 °C for 1 min, and 97 °C for 10 s. Upon completion of qRT-PCR amplification, raw data were accessed using the standard curve-based absolute quantification method in the software of the real-time fluorescence quantitative PCR system (LightCycler480 II, Roche, Basel, Switzerland). Based on the concentrations, dilution ratios, and volumes of diluted standards, copy numbers in the standard wells were calculated and input into the standard curves for automatic calculation of the expression levels of *CXCR4, Gnαq, PLCβ1, MMP1*, and *MMP3* mRNA in the spleen and joints of rats.

2.9. Measurement of protein levels of the CXCR4-Gnαq-PLCβ signaling pathway in the spleen and joints of rats

Frozen splenic and joint tissues were ground in liquid nitrogen, added to a mixture containing 1 mL of lysis buffer (Cat # 20220415, Solarbio Sci. & Tech. Co., Ltd.), 10 μL of protease inhibitor (Cat # A8260, Solarbio Sci. & Tech. Co., Ltd.), and 10 μL of PMSF (Cat #P0100, Solarbio Sci. & Tech. Co., Ltd.), incubated on ice for 30 min, and subsequently centrifuged at 12 000 r/min and 4 °C for 30 min. Total protein concentration and purity were measured in the supernatant using the BCA Protein assay kit (Cat # PC0020, Solarbio Sci. & Tech. Co., Ltd.). Equal amounts (30 μg) of protein were subjected to SDS-PAGE electrophoresis in a 5 % stacking gel at 80 V for 30 min and a 10 % resolving gel at 160 V for 45 min. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Cat # AR0136, Boster Bio. Tech. Co., Ltd., Wuhan, China) by wet transfer at 230 mA for 90 min and blocked with 5 % skim milk powder on a shaker at 20 °C for 2 h, Membranes were separately incubated at 4 °C overnight with the following antibodies: CXCR4/HRP rabbit polyclonal antibody (40 kDa, 1:1000, Cat # bs-1011R, Biosynthesis Bio. Co., Ltd., Beijing, China), GNAQ/HRP rabbit polyclonal antibody (42 kDa, 1:1000, Cat # bs-6152R, Biosynthesis Bio. Co., Ltd.), phospholipase C beta 1/HRP rabbit polyclonal antibody (134 kDa, 1:1000, Cat # bs-6976R, Biosynthesis Bio. Co., Ltd.), MMP1/HRP rabbit polyclonal antibody (54 kDa, 1:1000, Cat # bs-0424R, Biosynthesis Bio. Co., Ltd.), MMP3/HRP rabbit polyclonal antibody (52 kDa, 1:1000, Cat # bs-0413R, Biosynthesis Bio. Co., Ltd.), and anti-GAPDH polyclonal antibody (36 kDa, 1:2000, Cat #K110496P, Solarbio Sci. & Tech. Co., Ltd.). Goat anti-rabbit IgG/HRP (1:3000, Cat # SE134, Solarbio Sci. & Tech. Co., Ltd.) was added to membranes and incubated at 20 °C for 2 h. After incubation, thorough washing was performed thrice with TBST [140 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.05 % Tween20 (v/v)] for 10 min each time, and immunoblots were visualized using a ready-to-use ultra-sensitive ECL chemiluminescent substrate (Cat # AR1172, Boster Bio. Tech. Co., Ltd.). Exposure and image development were performed on a gel imaging system (Universal Hood II, Bio-Rad Laboratories, Inc., Singapore, Singapore), and grayscale values were analyzed in Image-Pro Plus 7.0 (Media Cybernetics Co. Ltd., Rockville, MD, USA).

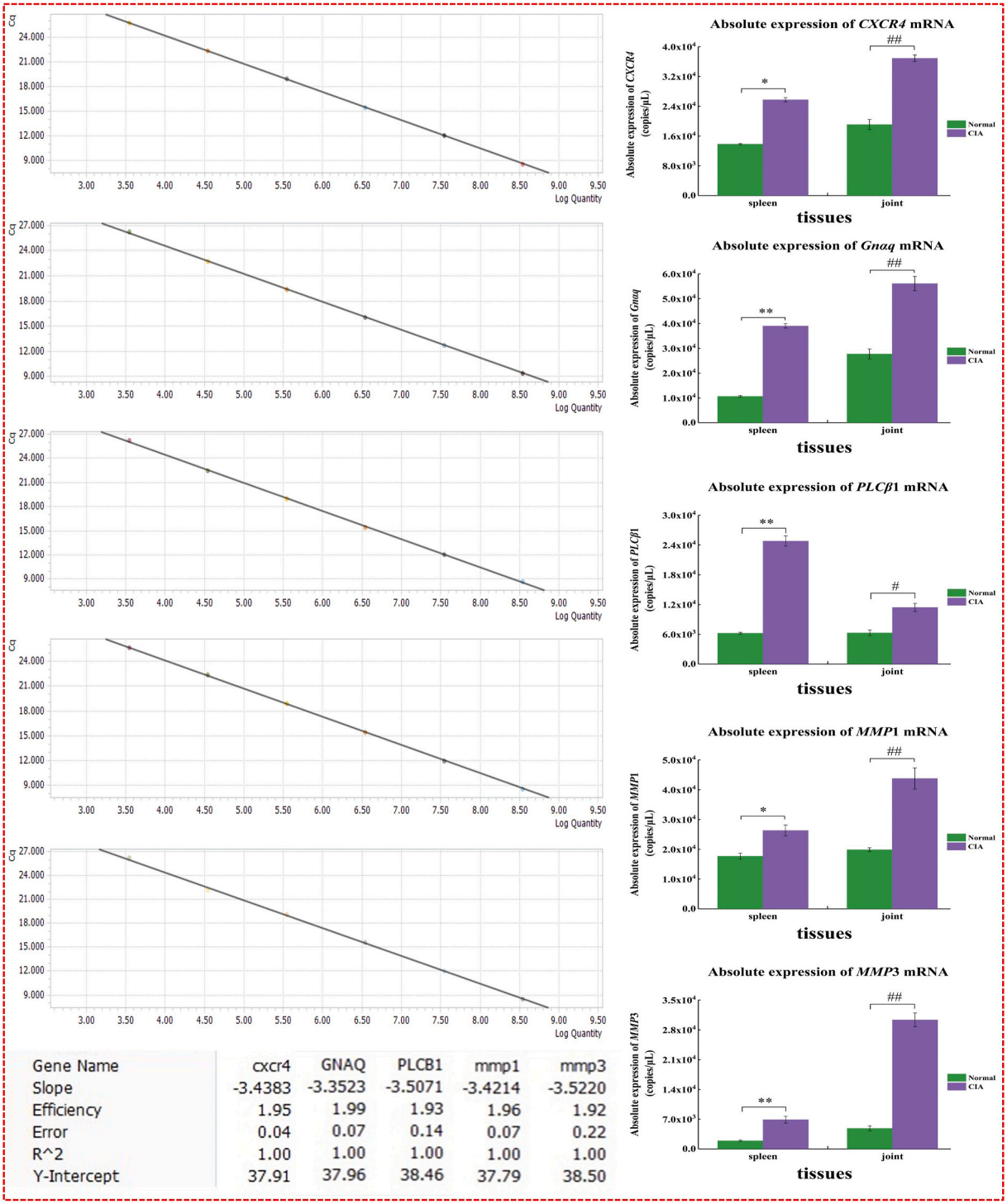
2.10. Immunohistochemical analysis of the CXCR4-Gnαq-PLCβ signaling pathway in the spleen and joints of rats

Spleen and joint slices were routinely dewaxed in xylene and dehydrated in a graded series of alcohol, deactivated in 3 % H_2O_2 at 20 °C for 5 min, rinsed with PBS, subjected to antigen retrieval with EDTA at 95 °C for 10 min, cooled to 25 °C, rinsed with PBS again, and blocked with 5 % BSA at 37 °C for 30 min. The following prediluted antibodies were separately added dropwise: CXCR4/HRP rabbit polyclonal antibody (1:150), GNAQ/HRP rabbit polyclonal antibody (1:150), phospholipase C beta 1/HRP rabbit polyclonal antibody (1:200), MMP1/HRP rabbit polyclonal antibody (1:150), MMP3/HRP rabbit polyclonal antibody (1:200); whereas, the control group received an equivalent amount of antibody diluent. Tissue slices were placed in a humid chamber at 4 °C overnight (12–16 h) and subsequently incubated at a constant temperature of 37 °C for 20 min. After incubation, the HRP-conjugated secondary antibody was added, and slices were incubated at 37 °C for 20 min, washed thrice with PBS for 5 min each time, subjected to color development with DAB for 5 min, washed thrice with distilled water for 3 min each time, counterstained with hematoxylin for 30 min, washed twice with water for 2 min each time, washed with hydrochloric acid (50 μL:100 mL) for 30 s, washed with ammonia water (150 μL:100 mL) for 7 min, rinsed with distilled water, cleared, mounted, and examined under imaging system (Molecular Imager® ChemiDoc™ XRS$^+$ System, Bio-Rad Laboratories Co., Ltd., Shanghai, China). Microscopic images were acquired using Image Pro Plus 7.0 (Media Cybernetics Co. Ltd., Maryland, USA).



(caption on next page)

Fig. 1. Phenotypic parameters of CIA rats compared with those of control rats. A. Changes in the body weight and arthritis scores of rats; B. Pathological changes in the joints and spleen of rats; C. Serum levels of inflammatory factors (interleukin-1β, −4, −6, and tumor necrosis factor-alpha) in control and CIA groups.



Gene Name	cxcr4	GNAQ	PLCB1	mmp1	mmp3
Slope	-3.4383	-3.3523	-3.5071	-3.4214	-3.5220
Efficiency	1.95	1.99	1.93	1.96	1.92
Error	0.04	0.07	0.14	0.07	0.22
R^2	1.00	1.00	1.00	1.00	1.00
Y-Intercept	37.91	37.96	38.46	37.79	38.50

Fig. 2. Standard curve, absolute expression of *CXCR*4-*Gnaq*-*PLCβ* signaling pathway-related genes in the spleen and joints of rats in control and CIA groups. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. spleen of control rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. joint of control rats.

2.11. Data processing and image analysis

All data were analyzed in SPSS 24.0 (IBM, Armonk, NY, USA). Statistical analysis was performed using analysis of variance (ANOVA), and multiple comparisons were performed using Duncan's multiple-range test. Graphs were plotted in Origin 2017 (OriginLab, Northampton, MA, USA). Data are expressed as the mean ± standard error (S.E.). Immunohistochemical staining results were processed in Image Pro Plus 7.0. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Phenotypic parameters in CIA rats

We observed that during the period of arthritis development, rats immunized with bovine type II collagen did not show a significant increase in body weight ($P > 0.05$), whereas the body weight of nonimmunized rats was significantly increased ($P < 0.05$). Joint scoring for all limbs of rats in the CIA group revealed that arthritis development was highly time-dependent. We detected that the maximum arthritis score occurred in week 3, and was significantly higher than those at other time points ($P < 0.01$) (Fig. 1A). Pathological examination of the spleen and joints of the two groups (Fig. 1B) indicated that the control group did not exhibit abnormal changes in the spleen and possessed intact articular structures, whereas the CIA group exhibited obvious hyperplasia in the lymphoid white pulp and main germination centers of the spleen. Rats in the control group had smooth and intact articular surfaces covered with synovial cells, whereas rats in the CIA group showed narrowing of joint cavities and inflammatory cellular infiltration on articular surfaces. ELISA assays (Fig. 1C) revealed a highly significant increase in the levels of serum IL-1β, IL-6, and TNF-α ($P < 0.01$) and a significant increase in the level of serum IL-4 ($P < 0.05$) in the CIA group compared with those in the control group. These results indicated the successful establishment of the CIA rat model.

3.2. Levels of mRNA expression of the CXCR4-Gnαq-PLCβ signaling pathway in the spleen and joints of rats

Analysis of the absolute quantification standard curves of *CXCR*4, *Gnαq*, *PLCβ*1, *MMP*1, and *MMP*3 constructed using plasmid standards (Fig. 2) revealed their good linear relationships with amplification efficiencies of 1.95, 1.99, 1.93, 1.96, and 1.92, respectively. The R^2 values (linear fit) were 1.00, demonstrating that the absolute quantification standard curves established in the present study satisfied the testing requirements. We found that the CIA group exhibited a significant increase in the levels of expression

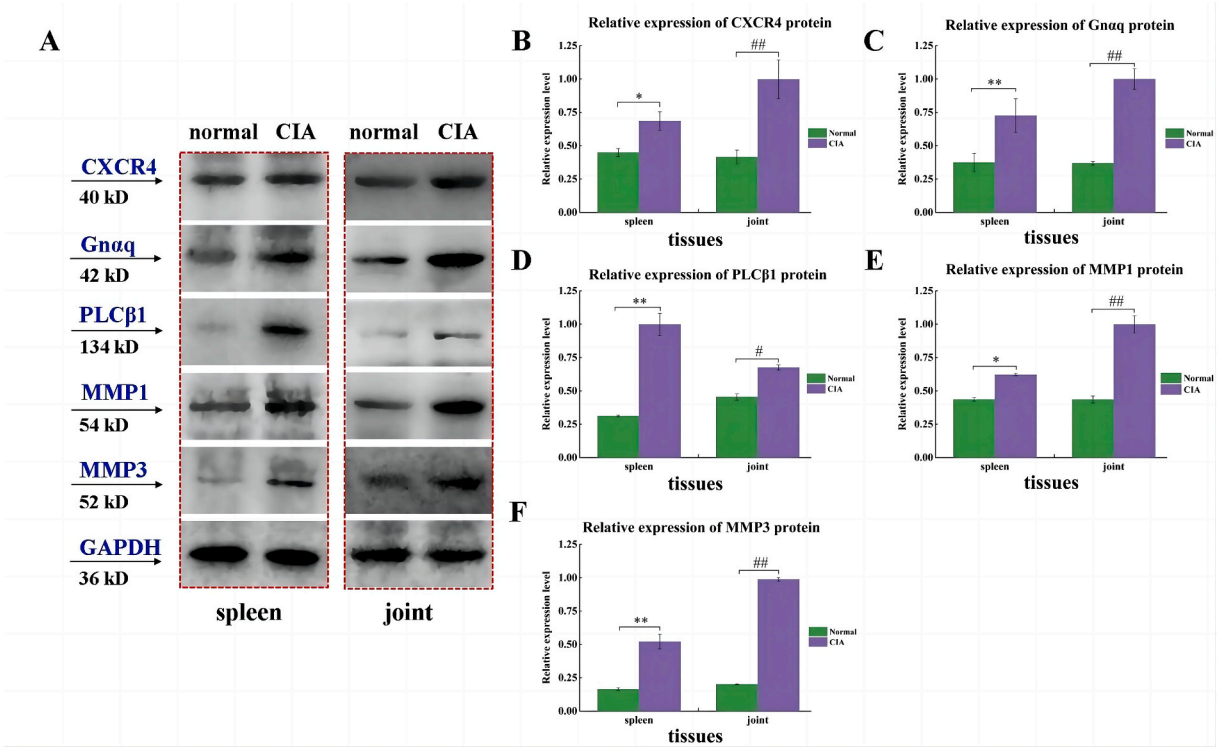


Fig. 3. Western blotting and quantitative analysis of CXCR4-Gnαq-PLCβ signaling pathway-related proteins in the spleen and joints of rats in control and CIA groups. A. Western blotting results in control and CIA rats; B–F. Relative expression of CXCR4, Gnαq, PLCβ1, MMP1, and MMP3 in the spleen and joints of control and CIA rats. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. spleen of control rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. joint of control rats.

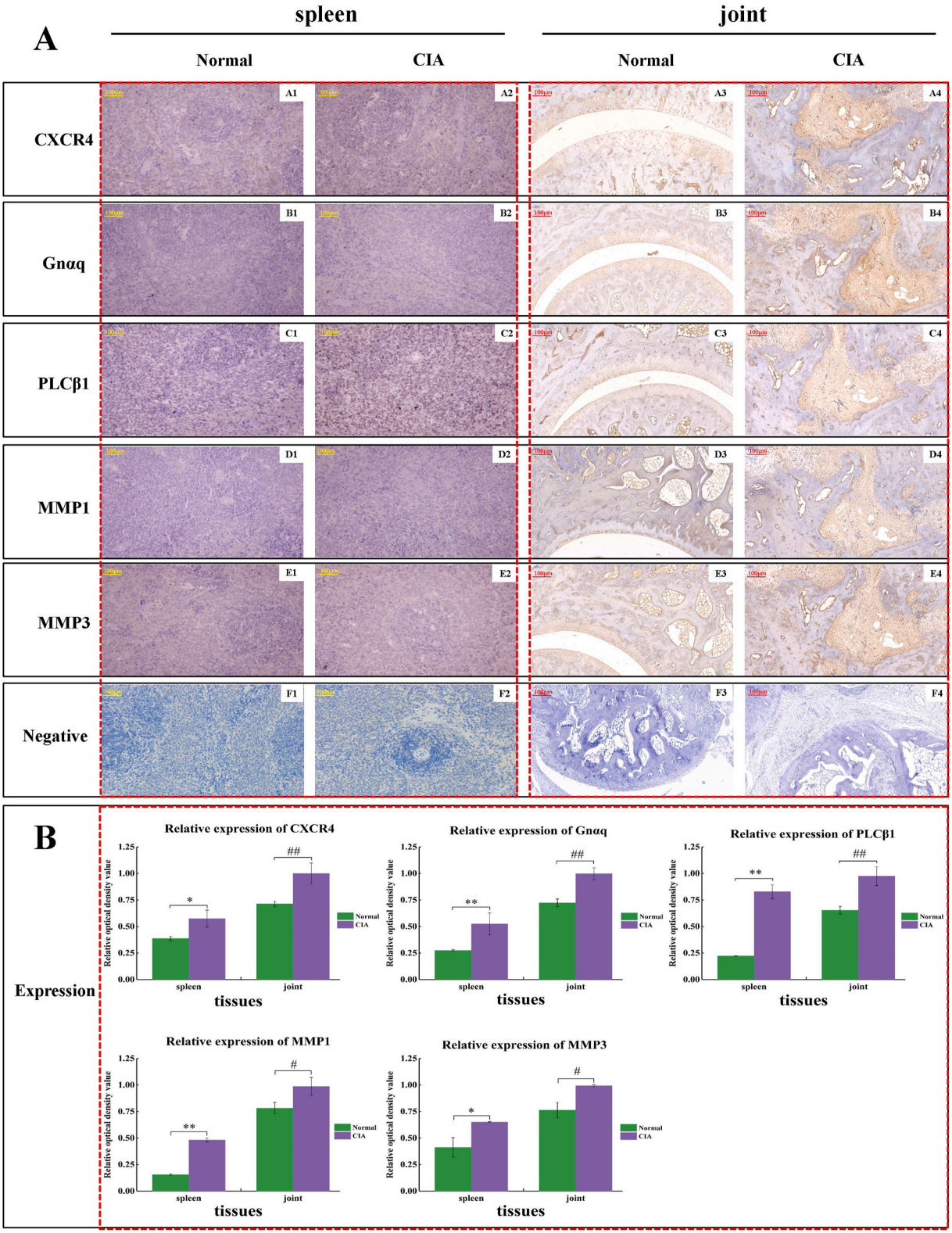


Fig. 4. Representative images of immunostaining of CXCR4-Gnαq-PLCβ signaling pathway-related proteins in the spleen and joints of rats in control and CIA groups. A. Representative images of immunostaining of CXCR4-Gnαq-PLCβ signaling pathway-related proteins. B. Relative optical density values of CXCR4-Gnαq-PLCβ signaling pathway-related proteins. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. spleen of control rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. joint of control rats. Scale bar, 100 μm. The experiment was repeated thrice.

of splenic *CXCR*4 and *MMP*1 mRNA ($P < 0.05$) and a highly significant increase in the levels of expression of splenic *Gnαq*, *PLCβ*1, and *MMP*3 mRNA ($P < 0.01$) compared with those in the control group. We observed that the joints of rats in the CIA group showed a highly significant increase in the expression of *CXCR*4, *Gnαq*, *MMP*1, and *MMP*3 mRNA ($P < 0.01$) and a significant increase in that of *PLCβ*1 mRNA ($P < 0.05$) compared with those in the contol group. These results, although preliminarily, indicated that the *CXCR*4-*Gnαq*-*PLCβ* signaling pathway participates in the onset and development of RA by mediating the formation of inflammatory cytokines.

3.3. Levels of protein expression in the CXCR4-Gnαq-PLCβ signaling pathway in the spleen and joints of rats

The western blotting results showed that CXCR4-Gnαq-PLCβ signaling pathway-related proteins were expressed in the spleen and joints of rats in both experimental groups (Fig. 3 A). Measurement of the levels of expression of CXCR4-Gnαq-PLCβ signaling pathway-related proteins in the spleen and joints of rats revealed that the CIA group had a significant increase in splenic CXCR4 and MMP1 expression levels ($P < 0.05$) (Fig. 3 B and E) and a highly significant increase in splenic Gnαq, PLCβ1, and MMP3 expression levels ($P < 0.01$) compared with the normal group (Fig. 3C, D and F). We also detected a highly significant increase in the expression of CXCR4, Gnαq, MMP1, and MMP3 ($P < 0.01$) and a significant increase in that of PLCβ1 ($P < 0.05$) compared with those in the control group. These findings were consistent with the results of the *CXCR*4-*Gnαq*-*PLCβ* signaling pathway-related mRNA expression, further demonstrating that the *CXCR*4-*Gnαq*-*PLCβ* signaling pathway participates in the inflammatory responses of RA and mediates the formation and exacerbation of inflammatory cytokines in RA, with the regulatory mechanisms likely being systemic in nature.

3.4. Immunohistochemical evaluation of the CXCR4-Gnαq-PLCβ signaling pathway in the spleen and joints of rats

We determined (Fig. 4) that proteins related to the CXCR4-Gnαq-PLCβ signaling pathway were expressed to varying levels in the spleen and joints of rats in the two groups. These proteins were primarily localized to the red and white pulp regions in the spleen and stromal, endothelial, and subdifferentiated synovial cells in joints. We found that compared with the control group, the CIA group showed a significant increase in the expression of splenic CXCR4 and MMP3 ($P < 0.05$) and a highly significant increase in splenic Gnαq, PLCβ1, and MMP1 expression ($P < 0.01$). The joints of CIA rats showed a significant increase in the expression of CXCR4, Gnαq, and PLCβ1 ($P < 0.01$) and a highly significant increase in that of MMP1 and MMP3 ($P < 0.05$).

4. Discussion

Although the pathogenic mechanisms of RA have not yet been fully elucidated, the cytokine network plays a crucial role in synovial inflammation and bone destruction in RA, serving as a key factor in the persistence, prolongation, and progression of lesions. These cytokines primarily originate from macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS), mainly including the following: (1) proinflammatory cytokines such as TNF-α, IL-1β, and chemokines/receptors CXCR4/12 [16]; (2) cytokines with anti-inflammatory effects, such as MMPs [17]. The main signaling pathways that regulate RA are significantly associated with the Wnt signaling pathway, mitogen-activated protein kinase (MAPK) signaling pathway, JUN transcription factor family (JUN), Jun B proto-oncogene, interleukin-1 receptor I (IL1R1), and CXCR4-Gnαq-PLCβ signaling pathway. Therefore, the increased expression of these genes in RA indicates them as potential targets for RA treatment [18].

The proportion of memory T-cells expressing CXCR4 in the synovium is increased in RA. CXCR4 enters the site of inflammation through the recruitment of activated/memory T-cells, suggesting the participation of CXCR4 in the onset of RA [19,20]. Previous studies have found that Gnαq is critical for autoimmunity and plays a key role in the differentiation of regulatory B-cells (Bregs) [15]. Experiments on the apoptosis of peripheral blood lymphocytes (PBLs) of patients with RA further confirmed that the expression of Gnαq mRNA in PBLs is closely associated with RA disease activity and rheumatoid factor (RF) titer. This suggested that *Gnαq* may participate in the onset and progression of RA by regulating the survival and death of lymphocytes. PLCβ is the most renowned downstream effector molecule in the Gnαq pathway. Activated PLCβ serves as a core enzyme in the canonical pathways of the Gq/11 family, catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to release inositol trisphosphate (IP3) and diacylglycerol (DAG). This pathway has been widely studied as a hallmark signaling pathway transduced by Gnαq signaling [21]. Gnαq activates the PLC family and chemokines stimulate inflammatory cells, thereby regulating the entry of extracellular calcium and affecting the expression of downstream effectors such as MMPs.

In the present study, we found that the arthritis scores of rats in the CIA group were significantly elevated ($P < 0.01$). Rats exhibited severe swelling and deformation of ankle joints, obvious limitations in mobility function, enlargement and fusion of the lymphoid white pulp and main germination centers of the spleen, severe damage to articular structures and cartilage surfaces, massive inflammatory cell infiltration on articular surfaces, and disappearance of physiological joint cavities. The serum expression of the inflammatory cytokines IL-1β, IL-4, IL-6, and TNF-α was also significantly elevated. The mRNA and protein levels of expression of *CXCR*4, *Gnαq*, *PLCβ*1, *MMP*1, and *MMP*3 in the spleen and joints were also significantly increased ($P < 0.05$, $P < 0.01$), indicating that the CXCR4-Gnαq-PLCβ signaling pathway participated in the formation of inflammatory cytokines in CIA rats and mediated the exacerbation in the expression of inflammatory cytokines in RA, with the regulatory mechanisms likely being systemic in nature. Proteins related to the CXCR4-Gnαq-PLCβ signaling pathway were primarily localized to the red and white pulp regions in the spleen as well as in stromal, endothelial, and subdifferentiated synovial cells in the joints. This indicated that the normal physiological structures of the spleen and joints were damaged and obvious inflammatory responses were induced in CIA rats during the disease course of RA, ultimately affecting the functions of multiple organ systems in rats. Our results suggested that the aggravation of inflammatory responses in the body results in the abnormal activation of the CXCR4-Gnαq-PLCβ signaling pathway, thereby triggering the occurrence

of autoimmune responses.

5. Conclusion

The present study found that CXCR4 is dependent on Gnαq for inducing the expression of PLCβ1 and stimulation of secretion of inflammatory cytokines by inflammatory cells. This affects the expression of MMPs, which serve as downstream effectors, thereby promoting RA pathogenesis. Our results serve as a key reference for understanding the mechanisms of RA onset and development. However, further research is required to elucidate the exact regulatory mechanisms of the *CXCR4-Gnαq-PLCβ* signaling pathway in RA pathogenesis.

Ethics approval and participation consent

This study was reviewed and approved by the Institutional Animal Ethical Committee of Shanxi University of Chinese Medicine, with the approval number: AWE202302079.

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Patient consent for publication

Not applicable.

CRediT authorship contribution statement

Zhen Li: Writing – review & editing, Project administration. **Jingshu Liu:** Validation. **Xiaowei Sun:** Supervision, Funding acquisition. **Yutong Li:** Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data and materials used in this study are available upon request from the corresponding author (lz88@sxtcm.edu.cn).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27861.

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