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Blocking of YY1 reduce neutrophil infiltration by inhibiting IL-8 production via the PI3K-Akt-mTOR signaling pathway in rheumatoid arthritis

J. Lin,*†[1] Y. He,*†[1] B. Wang,*† Z. Xun,*† S. Chen,* Z. Zeng‡ and Q. Ou *†

*Department of Laboratory Medicine, the First Affiliated Hospital of Fujian Medical University, Fujian, †First Clinical College, Fujian Medical University, Fuzhou, and ‡Department of Hematology and Rheumatology, the First Affiliated Hospital of Fujian Medical University, Fujian, China

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Correspondence: Qishui Ou, Department of Laboratory Medicine, the first affiliated Hospital of Fujian Medical University, 20 Chazhong Road, Fuzhou, 350005, Fujian, China.
E-mail: ouqishui@fjmu.edu.cn

[1]These authors contributed equally to this work and should be considered co-first authors.

Summary

Our previous study revealed that Yin Yang 1(YY1) played an important part in promoting interleukin (IL)-6 production in rheumatoid arthritis (RA). However, whether YY1 has any role in regulation of IL-8 in RA remains unclear. YY1 and IL-8 expression in RA patients were analyzed by real-time polymerase chain reaction (PCR). Ingenuity pathway analysis (IPA) was used to analyze the signaling pathway involved in YY1-induced IL-8 production. The expression of YY1 and proteins involved in the pathway were detected by Western blot and enzyme-linked immunosorbent assay (ELISA). Migration of neutrophils was performed by chemotaxis assay. In this study, we found that high expression of IL-8 was positively associated with YY1 expression in RA. Blocking YY1 expression by YY1-short hairpin (sh)RNA lentivirus reduced IL-8 production. Mechanistically, we showed YY1 activated IL-8 production via the phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway. Further, using a co-culture system consisting of peripheral blood mononuclear cells (PBMC) and neutrophils, we found that migration of neutrophils would be inhibited by YY1 RNA interference. Finally, using the collagen-induced arthritis animal model, we showed that treatment with the YY1-shRNA lentivirus led to reduction of IL-8 levels and attenuation of inflammation and neutrophil infiltration *in vivo*. Our results reveal a role of YY1 involved in neutrophil infiltration in RA via the PI3K/Akt/mTOR/IL-8 signaling pathway. YY1 may be a new therapeutic target for treatment of RA.

Keywords: interleukin-8, neutrophils, rheumatoid arthritis, YY1

Introduction

Rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease that affects approximately 1% of the world's population, is characterized by neutrophil infiltration, joint destruction and function disability [1]. It is established that gene–environmental interaction contributes to the development of RA. Our previous study showed that transcription factor YY1 (Yin Yang 1) plays an important role in the pathogenesis of RA by regulating the production of interleukin (IL)-6, contributing to the process of RA [2]. However, due to the complex mechanism of RA, whether or not YY1 has any other role in the pathogenesis of RA is still unclear.

It is well known that neutrophils are the major leukocytes in RA joints and play a crucial role in the development of RA [3,4]. In RA, the mechanisms of neutrophil activation and migration are changed [5]. In the early stages of the disease, neutrophils first migrate to the synovial fluid (SF), where they phagocytose immune complexes, release protease-rich granules and produce large quantities of reactive oxygen species (ROS), leading eventually to the erosion and destruction of cartilage [6]. Migration and recruitment of neutrophils to the RA joints is a key pathogenesis of RA which is often mediated by chemokines [7]. IL-8 is a member of the CXC chemokine family, which acts as neutrophil chemotactic factor. It can be secreted by monocytes, macrophages, epithelial cells, endothelial cells and airway smooth muscle cells

[8]. Studies have shown that elevated IL-8 can promote the recruitment of neutrophils which contribute to the occurrence of chronic inflammation diseases, such as RA [4], psoriasis [9], inflammatory bowel disease [10] and palmoplantar pustulosis [11].

However, whether YY1 plays any role in the neutrophil infiltration in RA joints, and whether IL-8 contributes to this process, is unclear. In this study, we aimed to investigate the role of YY1 in IL-8 production and neutrophil infiltration in RA.

Materials and methods

Patients

One hundred RA patients [22 men and 78 women, aged 26–78 years, mean ± standard deviation (s.d.) 51 ± 12 years], 100 osteoarthritis (OA) and 100 healthy controls (HD) were enrolled into the study. Diagnosis of RA patients fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism criteria for RA [12]. Diagnosis of OA based on the OARSI guidelines [13] and the guidelines of the Chinese Rheumatology Association. The clinical characteristics of RA, OA and HD are shown in Table 1. The study was approved by the Institutional Medical Ethics Review Board of the First Affiliated Hospital of Fujian Medical University (IEC-FOM-013-1.0). Written or verbal consent were provided for the patients in the study.

Animals

Male DBA/1J mice, aged 6–8 weeks, were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Science. Mice were maintained under pathogen-free conditions. All experiments were performed according to the committee guidelines and approved by the Animal Experiment Center of the Fujian Medical University (SYXK (Fujian) 2012-0001).

Peripheral blood mononuclear cell (PBMC) isolation and culture

PBMC were isolated from the peripheral blood of RA and OA patients and HD controls according to the manufacturer's instructions. In brief, PBMC were isolated immediately by Ficoll (TBD Science, Tianjin, China) and resuspended in RPMI-1640 medium with 10% fetal bovine serum at a cell concentration of 10^5/ml. These PBMC were stimulated by Dynabeads human T-activator CD3/CD28 (Gibco, Carlsbad, CA, USA) and human IL-2 recombinant protein (eBioscience, San Diego, CA, USA). After 24 h, these activated PBMC were infected with YY1 over-expressing lentivirus (LV-YY1), YY1 short hairpin (sh)RNA lentivirus (LV-YY1-shRNA) or control lentivirus (LV-NC) [multiplicity of infection (MOI) = 10] and 5 μg/ml of polybrene. All the lentivirus was obtained from GenePharma (Shanghai, China). The primers used for lentivirus construction are shown in Supporting information, Table 1; 48 h later, cell cultures were collected for further use.

Establishment and treatment of collagen-induced arthritis (CIA)

CIA mice were induced as previously described [2]. On day 1, male DBA/1J mice were injected intradermally with 150 mg bovine type II collagen (Chondrex, Redmond, WA, USA) dissolved in 0·05 M acetic acid and emulsified in Freund's complete adjuvant. On day 21, 75 mg collagen II in Freund's incomplete adjuvant was administered to the mice by intradermal injection. Joint inflammation was evaluated from 28 days after the first immunization, with a scale of 1–4. The total score for each mouse was 16, based on four paws. When the CIA mice had an

Table 1. Demographic characteristics of rheumatoid arthritis (RA) patients, osteoarthritis (OA) patients and healthy donors

Clinical data	RA	OA	HD
Age, mean (range), years	51·05 (26–78)	53·25 (19–78)	51·79 (49–55)
Sex, no. female	78	66	64
Sex, no. male	22	34	36
WBC, mean ± s.d., 10^9/l	8·00 ± 2·43	7·36 ± 2·54	6·14 ± 1·56
RBC, mean ± s.d., 10^{12}/l	4·39 ± 0·41	4·43 ± 0·54	4·70 ± 0·44
PLT, mean ± s.d., 10^9/l	262·66 ± 63·18	254·15 ± 88·36	267·10 ± 63·67
HGB, mean ± s.d., g/l	128·70 ± 15·57	130·32 ± 16·31	141·70 ± 14·69
RDW, mean ± s.d., %	14·43 ± 1·70	13·76 ± 1·36	13·00 ± 0·80
ACPA, median (IQR), RU/ml	125·8 (151·8)	1 (0·7)	–
CRP, median (IQR), mg/l	10·25 (12·4)	12·6 (8·22)	–
RF, median (IQR), IU/ml	107 (172·95)	< 10·2 (0)	–
ESR, median (IQR), mm/h	25 (26·75)	20 (24·75)	–

RA = rheumatoid arthritis; OA = osteoarthritis; HD = healthy donors; ACPA = anti-cyclic citrullinated peptide antibodies; RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; IQR = interquartile range; s.d. = standard deviation.

arthritic score of 2–3, the mice were divided into two groups and treated with LV-YY1-shRNA or LV-NC, respectively, by tail intravenous injection, 4×10^7 transducing units (TU)/mouse.

Histopathology

CIA mice were killed 70 days post-immunization,. The knee joints were dissected and fixed in 10% phosphate-buffered formalin for 2 days, then decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for 15 days. The specimens were embedded in paraffin, and serial paraffin sections were cut throughout the joint by a microtome. The sections were stained with hematoxylin and eosin (H&E) and observed by light microscopy.

Real-time PCR analysis

Total RNA was extracted from cells, and real-time PCR was performed as previously described [14]. Briefly, total RNA was extracted from cells using a TransZol Up Plus RNA Kit (Transgen, Beijing, China). Messenger RNA (mRNA) was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Two-step real-time PCR was performed using SYBR Premix Ex Taq (Takara, Dalian, China), according to the manufacturer's instructions. The primers used in this study are shown in Supporting information, Table 1. Thermocycler conditions were as follows: initial holding at 95°C for 30 s, followed by a two-step PCR process that consisted of 95°C for 3 s and 65°C for 30 s for 40 cycles. Data were collected, and quantitative analysis was performed using an ABI Prism 7500 sequence detection systemwThe glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the endogenous control. Gene expression was then calculated as the difference in cycle threshold (ΔCt) between the target gene and GAPDH; relative expression of target genes was calculated as $2^{-\Delta Ct}$.

Western blot analysis

Protein immune blotting was performed as previously described [15]. Briefly, cell lysates were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford, MA, USA) at 90 V for 90 min. The expression of YY1 and the phosphorylation of phosphatidylinositol-3-kinase (PI3K) and Akt were analyzed using specific antibodies (Abcam, Cambridge, MA, USA). After washing with phosphate-buffered saline (PBS), the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G at room temperature for 1 h followed by washing with PBS. The target proteins were examined with an enhanced chemiluminescence (ECL) system (Beyotime, Shanghai, China) and visualized with ChemiDoc XRS+ System (Bio-Rad, Hercules, CA, USA). The grey value of the band was analyzed by Image J software.

ELISA detection

PBMC from RA patients were isolated and stimulated with IL-2 (10 IU/ml; R&D Systems, Minneapolis, MN, USA) and Dynabeads human T-activator CD3/CD28 (Gibco). After 24 h, the cells were infected with LV-YY1-shRNA, LV-YY1 or LV-NC (MOI = 10) lentivirus and 5 μg/ml of polybrene. Signaling pathway inhibitors, including 50 μM LY294002 (inhibitor of PI3K), 100 μM BAY 11-7082 [inhibitor of nuclear factor kappa B (NF-κB)], 50 μM SB203580 [inhibitor of mitogen-activated protein kinase (MAPK)], 10 μM SP600125 [inhibitor of Janus kinase (JNK)] and 50 μM rapamycin (inhibitor of mammalian target of rapamycin (mTOR)], were added to LV-YY1-treated PBMC. Cell supernatant was collected 48 h later and diluted (1 : 1000) for the measurement of IL-8 by ELISA, according to the manufacturer's recommendations (R&D Systems).

Neutrophil isolation

Neutrophils were isolated from peripheral blood of RA patients. Briefly, neutrophils were isolated under sterile conditions immediately by Ficoll (TBD Science). Residual erythrocytes were removed by hypotonic lysis, and the cells were resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum at a cell concentration of 10^5/ml. The neutrophil viability was confirmed using trypan blue staining.

Transwell migration assay

PBMC from RA patients, in the presence of Dynabeads human T-activator CD3/CD28 (Gibco) and human IL-2 recombinant protein (eBioscience, San Diego, CA, USA), resuspended at a concentration of 5×10^5 cells/ml in RPMI-1640 and 10% fetal bovine serum and treated with L V-YY1-shRNA or LV-NC lentivirus, were added to the lower chamber. Neutrophils isolated from RA patients were added to the top chamber; 8 h later the filters were fixed with 4% paraformaldehyde for 15 min. The cells remained on fixed filters and were observed under optical microscope and counted with Wright–Giemsa staining.

Microarray and ingenuity pathway analysis (IPA)

The IPA analysis was performed as in our previous study [2]. In brief, in the presence of Dynabeads human T-activator CD3/CD28 (Gibco) and IL-2 (10 IU/ml, R&D Systems), PBMC from RA patients were treated with LV-YY1-shRNA or LV-NC lentivirus for 4 h. Then, the PBMC was collected and total RNA from was extracted by the standard TRIzol method. Affymetrix array was

performed by Genechme Company Limited (Shanghai, China) using human primeview GeneChip. The signaling pathways involved were performed by IPA.

Statistical analysis

All the experiments were performed in triplicate or more. Data were presented as mean ± standard deviation (s.d.) or *n* (%). Analysis of variance was performed to determine differences among groups, and Student's *t*-test was used to analyze the differences between two groups. Comparisons of categorical variables were conducted using χ^2 testing. Correlation analyses were performed using Spearman's correlation test. Arthritic score of CIA mice was analyzed by repeated measured analysis of variance (ANOVA). A *P*-value less than 0·05 was considered as statistical difference for the statistical analyses. Statistical analyses were performed by the Statistical Package for the Social Sciences, version 22.0 (SPSS Inc, Chicago, IL, USA), Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) or GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results

YY1 and IL-8 levels are increased in RA patients

Our previous studies have shown that YY1 plays a critical role in RA by inducing IL-6 production. Many studies have found that IL-8 is also involved in the pathophysiology of RA [16,17]. In order to explore the role of YY1 in the production of IL-8, the expression of YY1 and IL-8 in peripheral blood derived from RA, OA patients and healthy donors was examined. The results showed that the mRNA expression and protein levels of YY1 (Fig. 1) and IL-8 (Fig. 1c) were increased in RA patients compared with OA patients and HD, and there was a significant positive association between YY1 and IL-8 expression (Fig. 1d).

Inhibited YY1 expression affected PI3K/AKT and mTOR signaling

IL-8 plays an important role in the development of RA, and many signaling pathways are involved in the regulation of IL-8. In the previous study, we observed that YY1 expression was positively correlated with IL-8 level; we then questioned whether there were signaling pathways involved in this procession. To clarify this, we treated PBMC from RA patients with lentivirus of LV-YY1-shRNA or LV-NC for microarray assay. The inhibitory effect of LV-YY1-shRNA was confirmed in our previous study [2]. Volcano plot analysis showed that expression of 757 genes showed a significant difference between LV-NC- and LV-YY1-shRNA-treated PBMC, including IL-6, YY1 and IL-8. The *x*-axis is the logarithmic transformation with 2 as base of the fold change, and the *y*-axis is the logarithmic transformation with 10 as base of the statistical

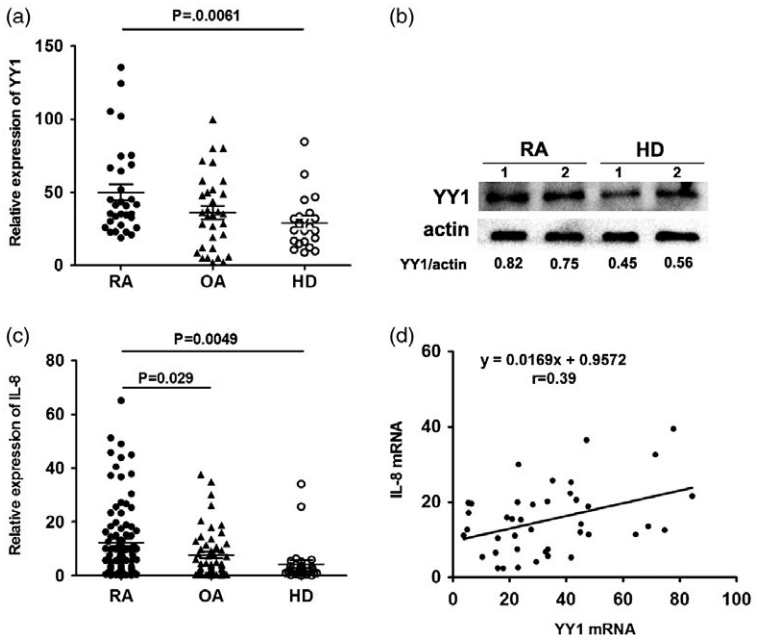

Fig. 1. Yin Yang 1 (YY1) and IL-8 were over-expressed in rheumatoid arthritis (RA) patients. (a) Relative expression of YY1 in RA patients (*n* = 32), osteoarthritis (OA) patients (*n* = 32) and healthy donors (*n* = 22) was detected by real-time polymerase chain reaction (PCR). (b) Protein level of YY1 in RA patients and health donors determined by Western blot. (c) Relative expression of interleukin (IL)-8 in RA patients (*n* = 32), OA patients (*n* = 32) and health donors (*n* = 22) detected by real-time PCR. (d) Correlation analysis between YY1 and IL-8 expression in RA patients.

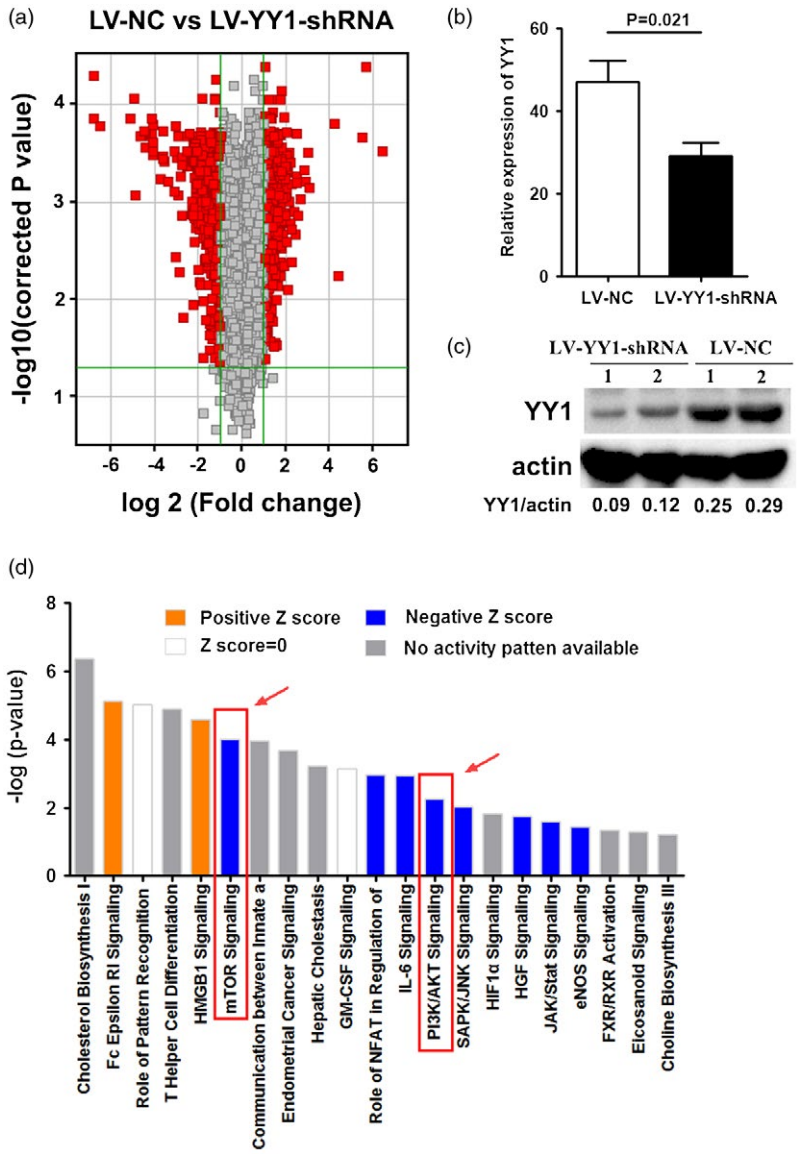

Fig. 2. Mammalian target of rapamycin (mTOR) signaling was restrained by Yin Yang 1 (YY1) blockade. (a) Volcano plot analysis of differences in gene distribution between lentivirus-control (LV-NC) or LV-YY1-short hairpin (shRNA)-treated peripheral blood mononuclear cells (PBMC). The *x*-axis is the logarithmic transformation with 2 as base of the fold change. The *y*-axis is the logarithmic transformation with 10 as base of the statistical significance. (b) mRNA expression of YY1 in LV-NC- or LV-YY1-shRNA-treated PBMC detected by real-time polymerase chain reaction (PCR). (c) Protein level of YY1 in LV-NC- or LV-YY1-shRNA-treated PBMC detected by Western blot. (d) The histogram of the signaling pathway shows the enrichment of the differentially expressed genes in the classical signaling pathway, and the analysis was shown by *Z*-score. Positive *Z*-score (yellow) indicated that the pathway was significantly activated. Negative *Z*-score (blue) indicated that the pathway was significantly suppressed.

significance (Fig. 2a). Moreover, decreased YY1 mRNA and protein expression was confirmed by real-time PCR and Western blot assay (Fig. 2). We further analyzed the changes in the signaling pathways by IPA, and the analysis result was shown by *Z*-score, showing that the PI3K/AKT and mTOR signal pathways were significantly inhibited in LV-YY1-shRNA-treated PBMC in RA patients (Fig. 2).

YY1 induced IL-8 production via the PI3K/Akt signaling pathway

It is well known that mTOR is a key kinase downstream of PI3K/AKT, which regulates tumor cell proliferation, growth [18,19] and inflammation [20,21], etc. As we found that the mTOR signaling pathway might take part in the production of IL-8 induced by YY1 in PBMC from RA

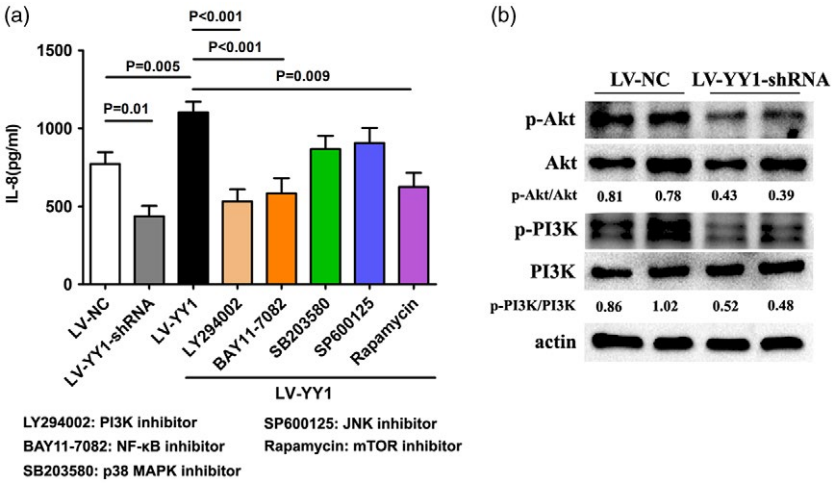

Fig. 3. Signaling pathways involved in Yin Yang 1 (YY1)-induced interleukin (IL)-8 production in lymphocytes of rheumatoid arthritis (RA). (a) Peripheral blood mononuclear cells (PBMC) isolated from RA patients were treated with lentivirus-control (LV-NC), LV-YY1-short hairpin (sh) RNA or LV-YY1. Then, 50 μM LY294002 (inhibitor of PI3K), 100 μM BAY 11-7082 [inhibitor of nuclear factor kappa B (NF-κB)], 50 μM SB203580 [inhibitor of mitogen-activated protein kinase (MAPK)], 10 μM SP600125 [inhibitor of Janus kinase (JNK)] and 50 μM rapamycin [inhibitor of mammalian target of rapamycin (mTOR)] was added to LV-YY1-treated PBMC. After 48 h, cell supernatant was collected for detection of IL-8 by enzyme-linked immunosorbent assay (ELISA). (b) Western blot analysis of the PI3K, Akt and phosphorylated PI3K, Akt expression in PBMC treated with LV-NC or LV-YY1-shRNA. The grey value of p-Akt/Akt and phosphatidylinositol-3-kinase (p-PI3K)/PI3K was calculated by Image J.

patients, we next detected several downstream signaling pathways of YY1 using known inhibitors of several pathways. The results showed that PI3K, NF-κB and mTOR inhibitors significantly inhibit the production of IL-8 in LV-YY1-treated PBMC from RA patients (Fig. 3a). To further validate the signaling pathway in LV-YY1-shRNA-treated cells, a Western blot assay was performed for detection of PI3K/Akt phosphorylation protein levels. The results showed that the phosphorylation of PI3K and Akt protein was significantly decreased in YY1 blocking PBMC (Fig. 3). Therefore, we believe that interfering with the expression of YY1 in lymphocytes will inhibit the release of IL-8 through the PI3K-Akt-mTOR signaling pathway.

Blocking of YY1 reduced inflammation in CIA mice

As we found that YY1 induced IL-8 production via the PI3K-Akt-mTOR signaling pathway *in vitro*, we determined whether YY1 plays a role in joint inflammation *in vivo*. We injected LV-YY1-shRNA and LV-NC, respectively, into CIA mice through the tail vein. The results showed that the inflammation score in the joints were significantly decreased in LV-YY1-shRNA-treated CIA mice (Fig. 4a). Compared to the LV-NC-treated mice, the mRNA and protein expression of YY1 in LV-YY1-shRNA-treated CIA mice was also decreased (Fig. 4). Consistently, we can see that the joints from LV-NC-treated mice were infiltrated with leukocytes. Moreover, the cartilage destruction and pannus formation in LV-NC-treated mice was more serious than that in LV-YY1-shRNA-treated mice (Fig. 4d). Furthermore, cytokine expression in the lymph node of LV-NC- or LV-YY1-shRNA-treated CIA mice was also examined. The results showed that the relative expression of IL-8, IL-6, IL-17A, IL-21 and IFN-γ were significantly decreased in LV-YY1-shRNA-treated CIA mice (Fig. 5). Taken together, these results suggested that blocking of YY1 ameliorated the inflammatory reaction of the CIA mice.

Blocking of YY1 suppressed neutrophil infiltration

IL-8 plays a major role in neutrophil migration through vascular endothelium and focal recruitment at inflammation sites. Our studies showed that blocking of YY1 could significantly decrease the expression of IL-8. To further identify whether YY1-blockage IL-8 secretion in PBMC has functional activity for reducing neutrophil migration, we established a mouse model of CIA and treated mice with LV-YY1-shRNA or LV-NC. The joints of the mice were obtained and cut into pathological sections for Wright–Giemsa staining. The microscopic results showed that the number of neutrophil infiltrations decreased significantly in the LV-YY1-shRNA-treated CIA mice (Fig. a,b). In addition, chemotaxis assay results showed that LV-YY1-shRNA-treated PBMC significantly reduced migration of neutrophils isolated from peripheral blood of RA patients (Fig. 6). Based on these results, our data demonstrated that blocking of YY1 activity with LV-YY1-shRNA could reduce neutrophil infiltration and migration by down-regulated IL-8 production.

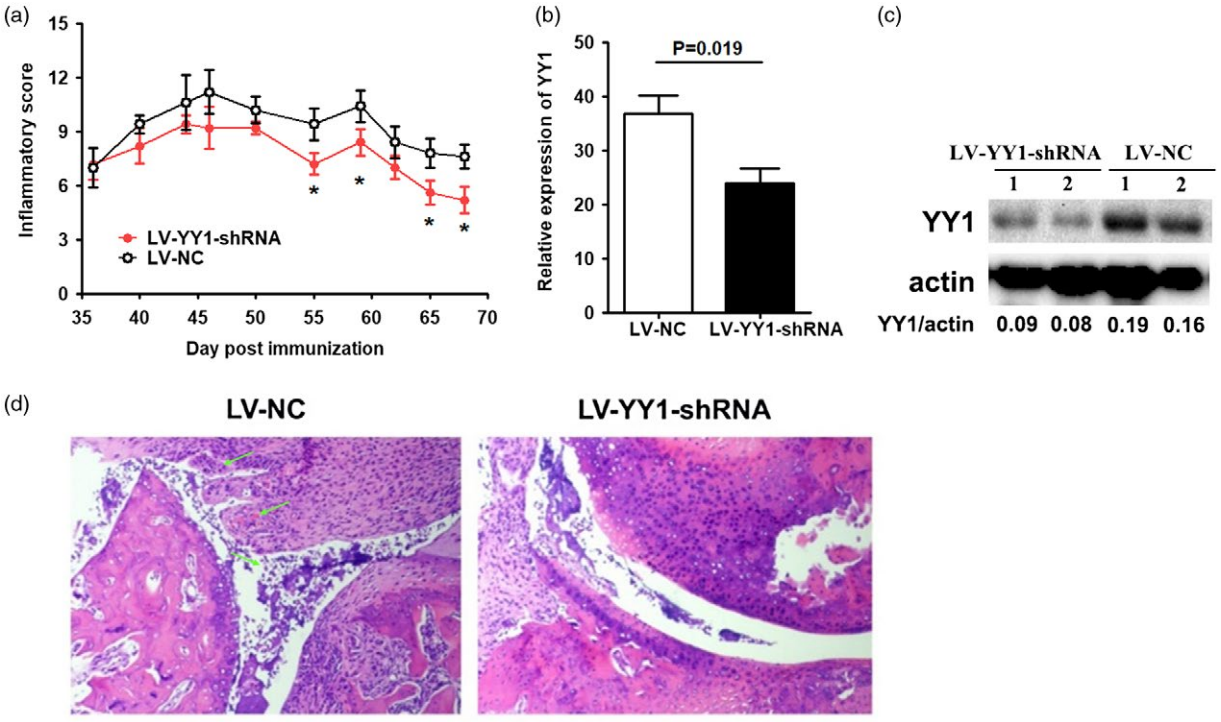

Fig. 4. Blocking of Yin Yang 1 (YY1) attenuated inflammation in collagen-induced arthritis (CIA) mice. (a) Clinical inflammatory score of CIA mice treated with lentivirus-control (LV-NC) ($n = 5$) or lentivirus (LV-)YY1-short hairpin (sh)RNA ($n = 5$). The inflammation score in the joints were significantly decreased in LV-YY1-shRNA-treated CIA mice ($P = 0{\cdot}029$). *$P < 0{\cdot}05$. (b,c) mRNA and protein expression of YY1 in LV-NC or LV-YY1-shRNA-treated CIA mice. (d) Histopathology of joint tissue sections of CIA mice treated with LV-NC ($n = 5$) or LV-YY1-shRNA ($n = 5$) by hematoxylin and eosin (H&E) staining. Original magnification ×200. Pannus formation, cartilage destruction and neutrophils infiltration is indicated by the green arrows.

Discussion

Although the pathogenesis of RA is unknown, accumulated evidence has shown that a large number of neutrophils were present in synovial fluid and deposited on the articular surface of RA patients, which can be activated by immune complexes (such as RF), and perform a completely different function than those isolated from healthy individuals [7]. On one hand, neutrophils possess the cytotoxic potential to promote the destruction of articular cartilage which, owing to their ability to release protease-rich granules, produce high amounts of ROS [5]. On the other hand, it can interact with fibroblast-like synoviocytes (FLS) and lymphocytes to promote intra-articular and peripheral inflammation and other immune responses characteristic of RA [22]. Many interventions used to treat RA have inhibitory effects on neutrophils. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids reduce neutrophil adherence, degranulation and ROS production. Disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate, induce neutrophil apoptosis and reduce neutrophil migration [7]. Tocilizumab reduces the number of neutrophils [23]. Animal experiments show that neutrophil-depleting antibody-treated mice have a slowly progressive disease, and neutrophil depletion can prevent joint inflammation if the antibodies are given before the induction of arthritis [6]. Moreover, some studies have shown that neutrophils and T helper type 17 (Th17) cells are key to the onset and perpetuation of RA. In early RA, increased levels of cytokines related with neutrophil activation and recruitment were found [6].

IL-8, a small protein family of 8–10 kDa, is an important cytokine that promotes the chemotaxis of neutrophils to the inflammation site of the joints. IL-8 exhibits pro-angiogenic activity and the ability to induce blood vessel formation and angiogenesis [24]. It is also a proinflammatory CXC chemokine associated with neutrophil chemotaxis and the pathogenesis of some chronic inflammatory diseases of neutrophil infiltration [11].

YY1 is a transcription factor with the ability to activate and inhibit gene transcription. It is a widely distributed transcription factor belonging to the GLI-Kruppel family of zinc finger protein [25]. Studies have shown that YY1 is over-expressed in many types of tumors, such as breast cancer [26], osteosarcoma [27], cervical cancer, hepatoblastoma, brain tumor [28], ovarian cancer, colon cancer, multiple myeloma [29], esophageal squamous cell

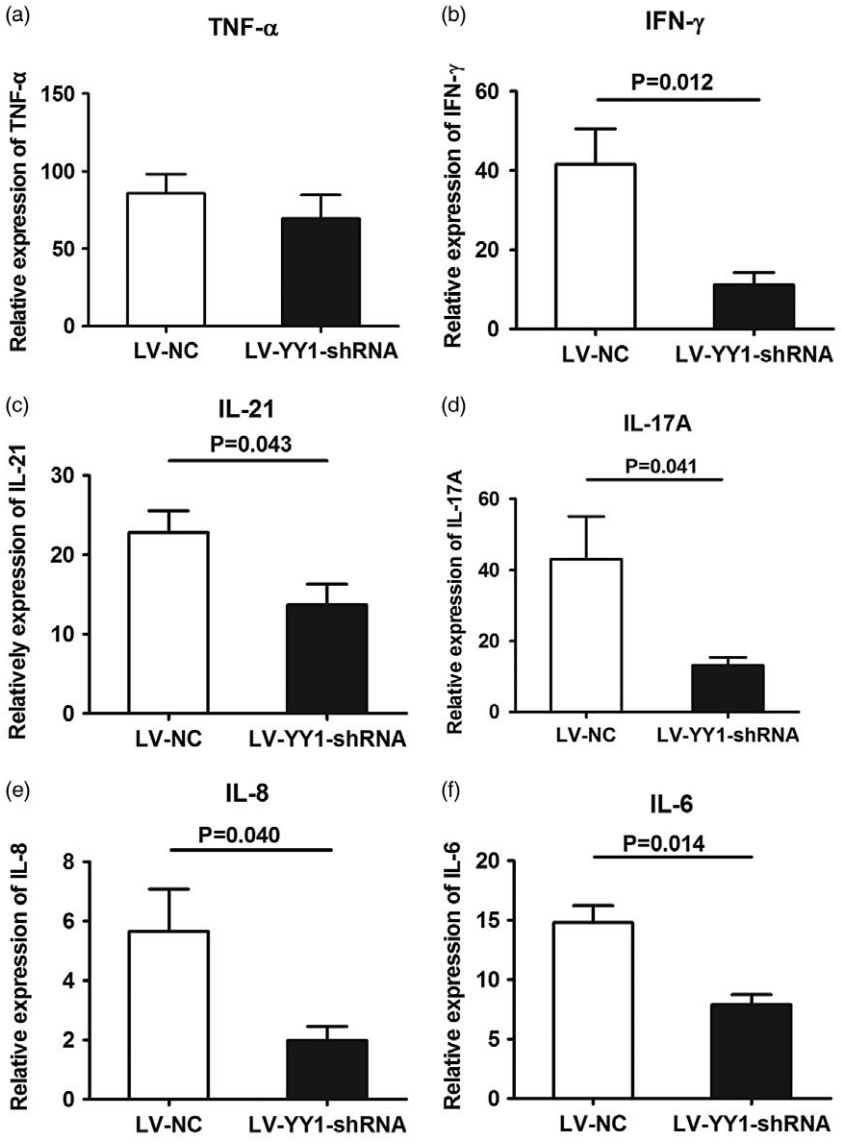

Fig. 5. Cytokine mRNA expression in lymph node of lentivirus-control (LV-NC) or LV-Yin Yang 1 (YY1)-short hairpin (sh)RNA-treated collagen-induced arthritis (CIA) mice. (a–f) Relative expression of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-21, IL-17A, IL-8 or IL-6 in lymph node of LV-NC (n = 5) or LV-YY1-shRNA (n = 5)-treated CIA mice.

carcinoma [30] and non-Hodgkin lymphoma [31], etc. Our previous studies have shown that YY1 can promote the pathogenesis of RA by promoting the production of Th17 cells [2]. However, whether YY1 is involved in IL-8 production and neutrophil infiltration remains unknown.

In this study, we first examined the expression of YY1 and IL-8 in RA patients. The results showed that they were significantly elevated in the peripheral blood of RA patients, and there is a positive correlation between YY1 and IL-8 levels. Similarly, in animal models of arthritis, we found that the level of joint inflammation was significantly decreased and the expression of YY1 and IL-8 in lymph nodes were also significantly decreased in LV-YY1-shRNA-treated CIA mice. These results indicated that YY1 has some relation to the expression of IL-8. However, the activity of YY1 has not been measured. Whether the activity of YY1 is related to IL-8 needs further study.

How does YY1 induce IL-8 production in RA? To clarify the signaling pathway through which YY1 promotes IL-8 production, a microarray assay was performed to identify the genes changed in LV-YY1-shRNA-treated cells. The signaling pathways involved were performed by IPA, and we found that the mTOR signaling pathway was inhibited in LV-YY1-shRNA-treated cells. As mTOR is a key kinase downstream of PI3K/AKT, which regulates tumor cell

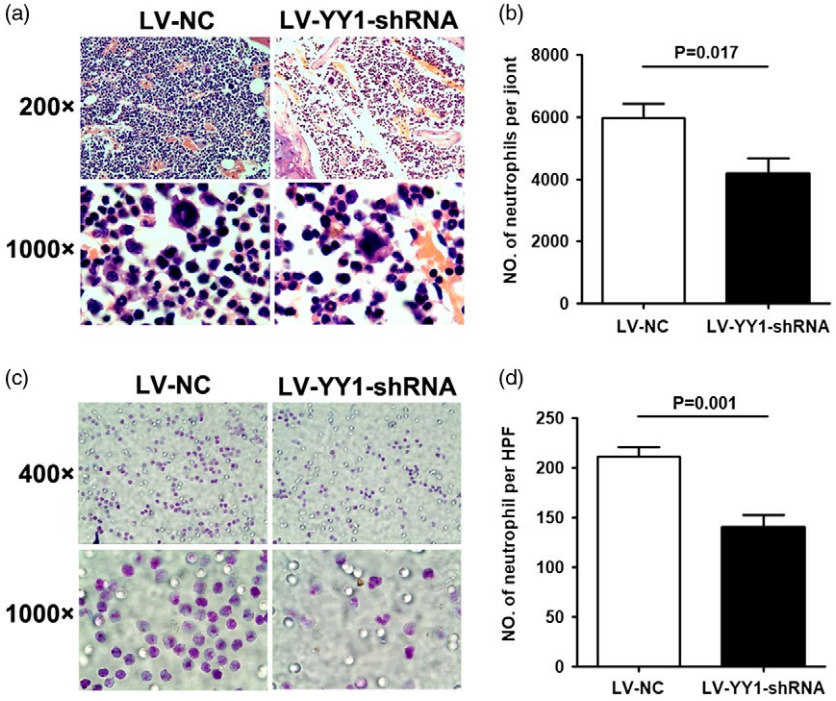

Fig. 6. Blocking of Yin Yang 1 (YY1) reduced neutrophil migration. (a) Neutrophils of joint tissue sections of collagen-induced arthritis (CIA) mice treated with lentivirus-control (LV-NC) (n = 5) or LV-YY1-short hairpin (sh)RNA (n = 5) by Wright–Giemsa staining. Original magnification ×200 and ×1000. Neutrophils are indicated by the green arrows. (b) Number of neutrophils per joint tissue sections of LV-NC- or LV-YY1-shRNA-treated CIA mice. (c) Migration of neutrophils in response to the culture supernatant from peripheral blood mononuclear cells (PBMC) treated with LV-NC (n = 5) or LV-YY1-shRNA (n = 5) by Wright–Giemsa staining. Original magnification ×400 and ×1000. (d) Number of migrated neutrophils in condition of LV-NC- or LV-YY1-shRNA-treated PBMC.

growth and proliferation [18,19] and is implicated in an increasing number of pathological conditions, including cancer [32], diabetes [33], neurodegeneration [34] and inflammation [20,21], etc., we evaluated five well-known pathways using known inhibitors to treat YY1-over-expressed lymphocytes and found that PI3K, NF-κB and mTOR signaling pathways have an impact on IL-8 production. Moreover, the phosphorylation of PI3K and Akt protein was significantly decreased in YY1 blocking lymphocytes. All the results revealed that YY1 can induce IL-8 production via the PI3K-AKT-mTOR signal pathway.

Further, in order to investigate whether the YY1-induced IL-8 production has some effect on neutrophil infiltration in RA, the neutrophil infiltration in CIA mice and *in vitro* were observed. We found that compared with LV-NC-treated CIA mice, the inflammation score and the neutrophil infiltration in the joints of LV-YY1-shRNA-treated CIA mice was significantly decreased. Next, we treated PBMC isolated from RA patients with LV-YY1-shRNA *in vitro*; Transwell migration experiments suggested that blocking of YY1 in PBMC could significantly reduce the migration of neutrophils. Taken together, these results suggest that inhibition of YY1 attenuates neutrophil infiltration by decreasing IL-8 production in RA.

Recently, a study showed that a novel NF-κB/YY1/miR-10a/NF-κB regulatory circuit promoted the secretion of NF-κB-mediated inflammatory cytokines, including IL-6 and IL-8 [35]. Moreover, another study found that YY1, a long non-coding RNA ANRIL binding transcription factor, was required for IL-6 and IL-8 expression under TNF-α treatment in coronary artery disease [36]. These findings were consistent with our results. However, it is not known if any other signaling pathway is involved in YY1-regulated IL-8 production. Our present study revealed that YY1 could promote neutrophil infiltration by regulating IL-8 production via the PI3K-AKT-mTOR signal pathway in RA, which may perfect the mechanism of YY1-regulated inflammation in RA. Combined with our previous studies [2], we believe that YY1 can promote Th17 cell production and neutrophil infiltration. Therefore, targeting YY1 may be the key strategy for future RA treatment.

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Disclosures

The authors declare that they have no conflicts of interest.

Author contributions

Y. H. and Z. Z. participated in the design of the study and drafted the manuscript. Z. X. and S. C. participated in analysis and interpretation of data. J. L. participated in the design of the study, performed the statistical analyses and revised the manuscript. Q. O. participated in the design of the study and revised the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website:

Fig. S1. The viability of PBMC cells treated with lentivirus. PBMC from RA patients were treated with LV-YY1-shRNA or LV-NC lentivirus, the viability of PBMC was detected by MTT assay. There is no significant difference between LV-NC, LV-YY1-shRNA and control groups.

Fig. S2. The viability of PBMC cells treated with lentivirus. PBMC from RA patients were treated with LV-NC, LV-YY1-shRNA or LV-YY1 lentivirus, the viability of PBMC was detected by MTT assay. There is no significant difference between LV-NC, LV-YY1-shRNA, LV-YY1 and control groups.

Fig. S3. Signaling pathway involved endogenous IL-8 production. PBMC from RA patients were stimulated with IL-2 (10IU/ml) and Dynabeads Human T-activator CD3/CD28. After 24hr, signaling pathway inhibitors 50μM LY294002, 100μM BAY 11-7082, 50μM SB203580, 10μM SP600125 (inhibitor of JNK) and 50μM rapamycin were added. 48hr later, cell supernatant was collected for the measurement of IL-8 by ELISA.

Table S1. The sequences of primers used in this study