ORIGINAL ARTICLES

Department of Orthopedics[1], Traditional Chinese Medicine of Shanxi Province Hospital, Xi'an city; The Second Department of Orthopedics[2], The Central Hospital of Weinan City, Weinan City; Department of Orthopedics and Traumatology[3], Department of Hand Surgery[2], Ruian People's Hospital, Ruian City; Department of Microsurgical Bone Repair[5], The Red Cross Hospital affiliated to Xi'an Jiao Tong University, Xi'an City, P.R. China

P54/nrb prompts rheumatoid arthritis progression mainly by transcriptionally activating NF-κB signaling

Qiang Li[1,#], Hongjun Guo[2,#], Huihui Li[3], Qi Zhu[4], Yang Liu[5,*]

Received Novemver 2, 2016, accepted December 9, 2016

[*]Corresponding author: Dr. Yang Liu, Department of microsurgical bone repair, The red cross hospital affiliated to Xi'an Jiao Tong University, Xi'an city, No.76, Nanguo Road, Shanxi Province, P.R. China, 710054.
gunr10010@163.com
[#]These authors contributed equally to this work.

Pharmazie 72: 260–264 (2017) *doi: 10.1691/ph.2017.6904*

In various tumors, aberrant expression of P54/nrb has been identified. However, the expression pattern and specific role of P54/nrb in rheumatoid arthritis (RA) has never been explored. Here, we first demonstrated that the expression of P54/nrb was markedly enhanced in the synovial tissues of RA patients. Functional study showed that P54/nrb could enhance the levels of inflammatory factors, including IL-1, IL-2, IL-6, IL-8 and TNFα. More importantly, we first found that overexpression of P54/nrb can induce the protein levels of P65, an important subunit of NF-κB. In contrast, knockdown of P54/nrb by RNAi significantly decreased the expression of NF-κB. Luciferase reporter assay and CHIP assay showed that P54/nrb could transcriptionally activate the expression of NF-κB, thereby enhancing pro-inflammatory responses. In summary, the expression of p54 was markedly increased in the synovial tissues of RA patients. Further study demonstrated that p54 could transcriptionally activate the expression of p65, an important NF-κB subunit, thereby enhancing the pro-inflammatory response.

1. Introduction

As a chronic auto-immune-based inflammatory disease, rheumatoid arthritis (RA) results in progressive joint degeneration, disability, and enhanced risk of cardiovascular complications, thereby causing mortality (Totoson et al. 2014; Swierkot et al. 2016). Over time, the immune cells infiltrate into the synovial lining, which is a major cause of hyperplasia and increased vascularisation (Ivashkiv and Hu 2003). An imbalance of pro-inflammatory and anti-inflammatory cytokines contributes to the dysregulation of cytokine synthesis, disturbances in the migration of immunocompetent cells and aberrant cell apoptosis (Mori et al. 2011; Ortiz et al. 2015). Therefore, it is of great importance to explore the underlying mechanism which modulates the pro-inflammatory/anti-inflammatory imbalance.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) participates in the regulation of multiple cellular processes, including cell growth, apoptosis, and inflammatory and immune responses (Verma 2004; Malemud 2013). Abnormal activation of NF-κB is reported to cause numerous auto-immune diseases, including arthritis (Mu et al. 2016). NF-κB-dependent gene expression is found to play a key role in the activity of many cytokines, including IL-1, IL-2, IL-6, IL-8, IL-12, TNFα, and monocyte chemotactic protein factor (MCP)-1 (Zhai et al. 2016; Zhang et al. 2016). However, the regulation mechanism of NF-κB activation is not fully understood.

P54/nrb is a RNA-binding molecule of 54 kDa, which is ubiquitously expressed with two RNA recognition motifs (Zhu et al. 2016). P54/nrb influences gene expression and differentiation in the nucleus, including transcriptional regulation, RNA splicing, and subnuclear body formation (Schiffner et al. 2011). In various tumors, aberrant expression of P54/nrb has been identified (Basu et al. 1997; Passon et al. 2012). However, its expression pattern and specific role of P54/nrb in RA has never been explored.

In the current study, we first demonstrated that the expression of P54/nrb was markedly increased in RA tissues compared with normal tissues. Further study showed that P54/nrb could transcriptionally activate NF-κB signaling thereby inducing auto-immune responses.

2. Investigations and results

2.1. Upregulation of p54 in synovial tissues of RA patients

Firstly, we explored the expression of p54 in the synovial tissues of RA patients. Western blot analysis demonstrated that p54 was markedly upregulated in the synovial tissues of RA patients (Fig. 1).

2.2. Activation of NF-κB signaling in synovial tissues of RA patients

Next, activation of NF-κB signaling was explored in the synovial tissues of RA patients. Western blot analysis demonstrated enhanced expression of p65 in in the synovial tissues of RA patients compared that of healthy controls (Fig. 2A). ELISA analysis demonstrated that the levels of IL-1, IL-2, IL-6, IL-8 and TNFα were also significantly enhanced in the serum of RA patients compared with that of normal controls (Fig. 2B).

2.3. Overexpression of p54 enhances the expression of pro-inflammatory cytokines

To further explore the role of p54 on the inflammatory response, we overexpressed p54 in SW982 cells. Western blot analysis demonstrated that transfection with ad-p54 into SW982 cells markedly enhanced the protein expression of p54 (Fig. 3A). More importantly, the levels of pro-inflammatory cytokines were explored. Real time PCR analysis demonstrated that overexpression of p54 significantly enhanced the level of IL-1, IL-2, IL-6, IL-8 and TNFα (Fig. 3B, C, D, E and F). These data indicated the pro-inflammatory effects of p54.

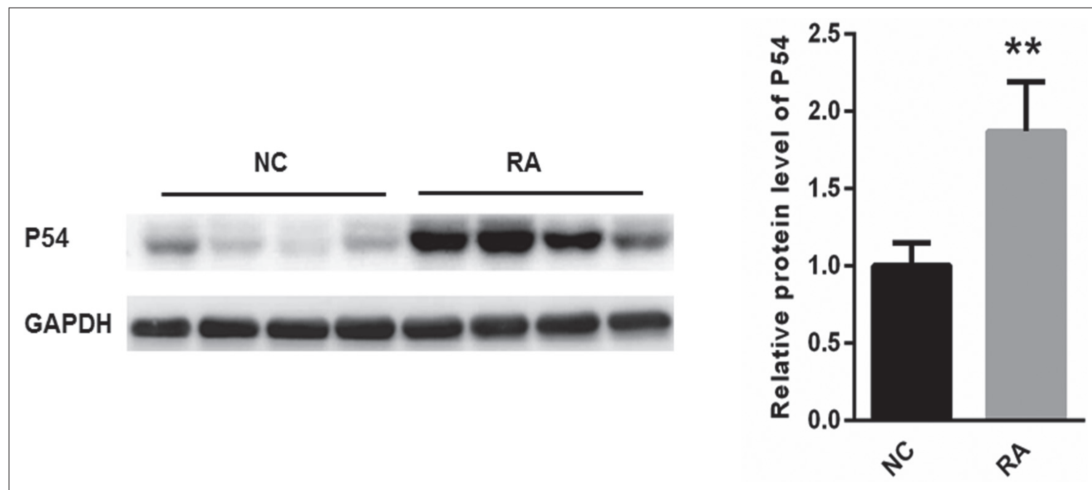

Fig. 1: Upregulation of p54 in synovial tissues of RA patients. *P<0.05, **P<0.01.

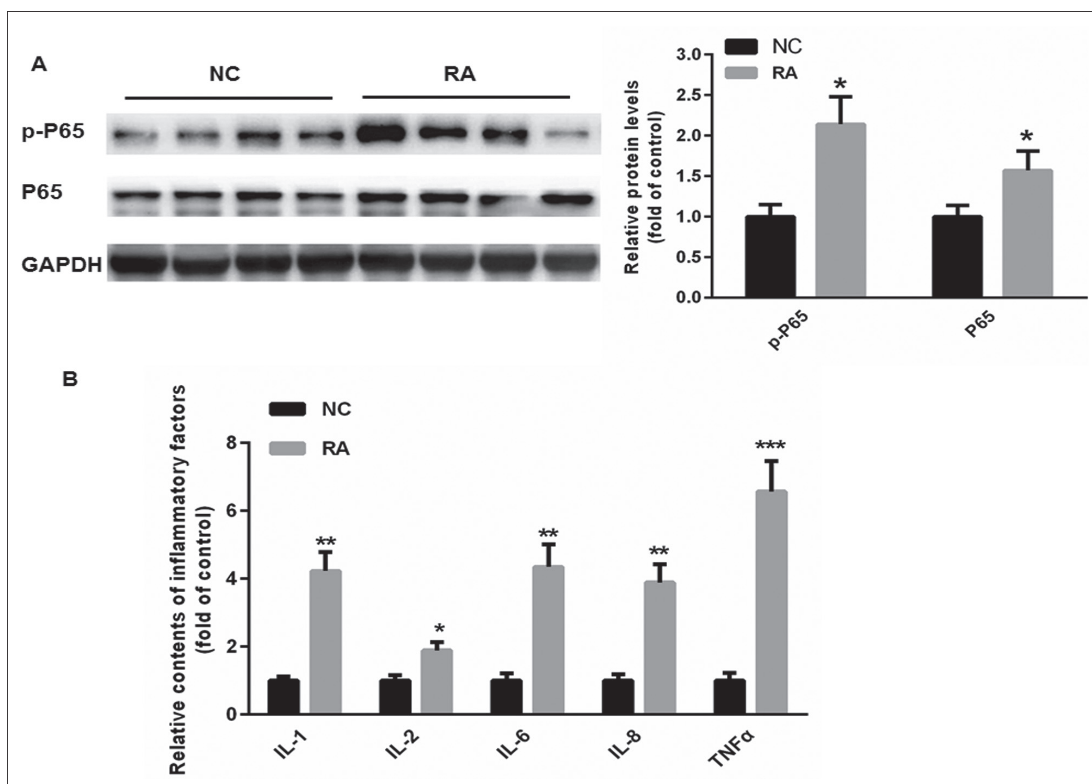

Fig. 2: Activation of NF-κB signaling in synovial tissues of RA patients. (B) Western blot analysis. (B) The levels of IL-1, IL-2, IL-6, IL-8 and TNFα analyzed by ELISA kits. *P<0.05, **P<0.01.

2.4. P54 activate the transcription of NF-κB

We then studied whether P54 could transcriptionally activate NF-κB signaling. In the promoter region of NF-κB P65 subunit, two putative binding sites of P54 were identified (Fig. 4A). Then, the promoter region containing the binding sites were cloned into pGL3 basic plasmid, pGL3-P65. Dual luciferase reporter assay demonstrated that overexpression of P54 significantly enhanced the relative luciferase activity of pGL3-P65 compared with blank vector (Fig. 4B). CHIP assay showed that silencing of P54 decreased the DNA fragments of P65 (Fig. 4C). In contrast, the DNA quantity of P54 was markedly increased when P54 was overexpressed as demonstrated by CHIP assay (Fig. 4D). At the transcription level, we also found that overexpression of P54 significantly enhanced the transcription of NF-κB (Fig. 4E).

2.5. P54 induces pro-inflammatory responses through NF-κB signaling

Based on the above results, we proposed that P54 mediated pro-inflammatory responses mainly through NF-κB signaling. Thus, in SW982 cells, a specific siRNA targeting NF-kB subunit, P65, was selected (Fig. 5A). In contrast, overexpression of P54-induced P65 upregulation was markedly abolished when si-P65 was transfected into SW982 cells (Fig. 5A). We also examined the levels of IL-1, IL-2, IL-6, IL-8 and TNFα in SW982 cells after transfection of ad-p54 and/or si-P65. ELISA analysis demonstrated that inhibition of P65 significantly suppressed the levels of IL-1, IL-2, IL-6, IL-8 and TNFα, even when P54 was overexpressed in SW982 cells (Fig. 5B, C, D, E and F). These data indicated that P54 mediated pro-inflammatory responses mainly through NF-κB signaling.

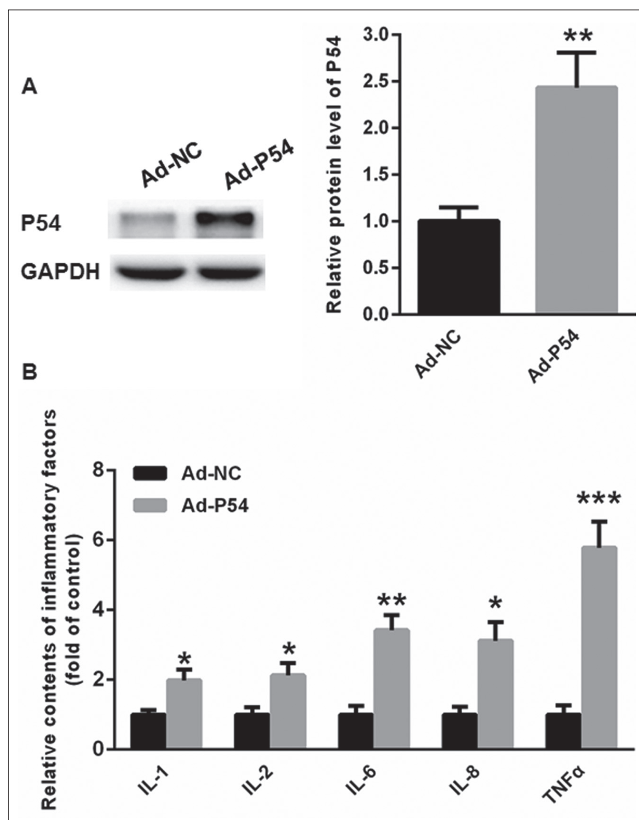

Fig. 3: Overexpression of P54 enhances the expression of pro-inflammatory cytokines. (A) Western blot analysis demonstrated that transfection with ad-P54 into SW982 cells markedly enhanced the protein expression of P54. (B) ELISA analysis of IL-1, IL-2, IL-6, IL-8 and TNFα levels in SW982 cells transfected with ad-P54 or ad-NC. *P<0.05, **P<0.01.

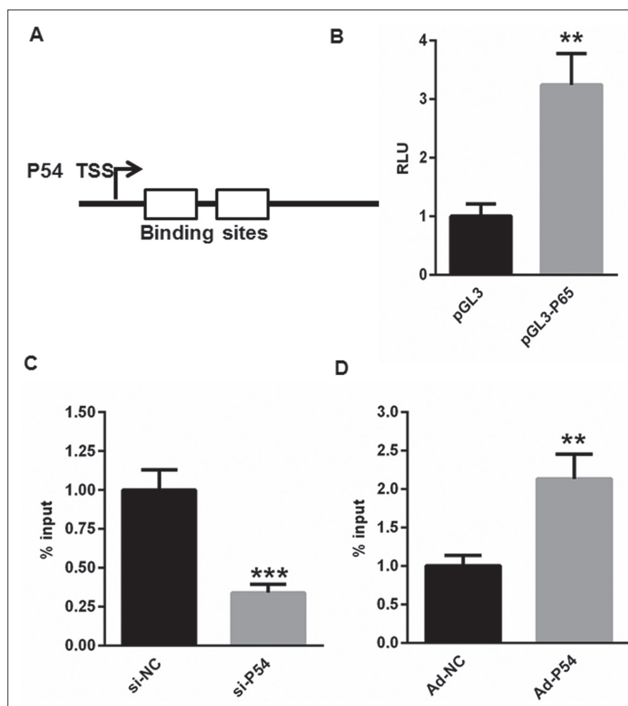

Fig. 4: P54 activate the transcription of NF-κB. (A) Two putative binding sites of P54 were identified in the promoter region of NF-κB P65 subunit. (B) Dual luciferase reporter assay. (C) CHIP assay showed that silencing of P54 decreased the DNA fragments of p65. (D) The DNA quantity of P54 was markedly increased when P54 was overexpressed as demonstrated by CHIP assay. (E) Overexpression of P54 significantly enhanced the transcription of NF-κB. *P<0.05, **P<0.01.

3. Discussion

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disorder that is characterized by substantial synovial proliferation and inflammation, affecting approximately 1% of the world's population (Pincus et al. 2001; Firestein 2003; Raptopoulou et al. 2007). The main characteristics of this disease are many kinds of cells infiltration, including immune cells (such as macrophage, T cells and B cells) and erosive cells, and aberrant fibroblast-like synoviocytes (FLS) proliferation (Koning 2015). It is reported that T cell immunity plays a key role in RA progression (Noort et al. 2015; Pruijn 2015; Yin et al. 2015). Studies have demonstrated the activation of inflammatory cells and enhanced expression of multiple cytokines in the tissues of RA patients (Cooles and Isaacs 2011; Pruijn 2015; Toussirot et al. 2015). In the synovial tissues and fluid of RA patients, inflammatory factors, including IL-6, IL-1 and TNF-α, were found to be markedly increased, thereby leading to chronic inflammation and joint destruction (Culshaw et al. 2011; Hinoi and Yoneda 2011). In the current study, we explored the levels of IL-1, IL-2, IL-6, IL-8 and TNFα in the serum of RA patients. In line with previous studies, significantly enhanced expression of IL-1, IL-2, IL-6, IL-8 and TNFα was found in the serum of RA patients compared to that of normal controls (Noort et al. 2015; Spurlock et al. 2015). More importantly, abnormal activation of NF-κB was identified in the tissues of RA patients. Under normal conditions, NF-κB locates in the cytoplasm which is inhibited by an inhibitor of NF-κB(IκB) (Mori et al. 2011; Swierkot et al. 2016). After exposure to external stimuli, IκB was phosphorylated and then degraded. Then, NF-κB translocates into the nucleus, thereby initiating the transcription of proinflammatory cytokines, including IL-6 and IL-8 (Verma 2004). It is well accepted that NF-κB plays a critical role in the progression of RA, therefore, it is the transcription factor is an enticing target for drug candidates. However, we still lack the knowledge about the regulation of NF-κB.

As a multi-functional nuclear protein, P54/nrb is found to be widely involved in various nuclear processes (Costa et al. 2016; Zhu et al. 2016). For instance, P54/nrb was reported to induce the expression of claudin-5 in normal blood-retinal barrier induction *in vivo* (Gerber et al. 1994; Keil et al. 2013). In addition, P54/nrb may increase gene expression through binding to the unmethylated promoter (Park et al. 2013). However, no study has been focused on the expression of P54/nrb in the synovial tissues of RA patients. In the current study, for the first time, we demonstrated that the expression of P54/nrb was markedly enhanced in the synovial tissues of RA patients. Functional study showed that P54/nrb could enhance the levels of inflammatory factors, including IL-1, IL-2, IL-6, IL-8 and TNFα. More importantly, we first found that overexpression of P54/nrb can induce the protein levels of NF-κB. In contrast, knockdown of p54/nrb by RNAi significantly decreased the expression of NF-κB. Our work provides novel evidence that p54/nrb is a positive regulator of NF-κB, thereby enhancing pro-inflammatory responses.

In conclusion, we observed, for the first time, that the expression of p54 was markedly increased in the synovial tissues of RA patients. Further study demonstrated that p54 could transcriptionally activate the expression of p65, an important NF-κB subunit, thereby enhancing the pro-inflammatory response. Our study may contribute to the development of novel inflammation targets for RA treatment.

4. Experimental

4.1. Patients and synovial tissues

Synovial biopsy specimens were obtained by needle arthroscopy from 58 patients who had clinically active arthritis of at least one knee joint. None of the patients had been treated with biological therapy at the time of inclusion. Arthroscopy was performed under diagnostic and/or therapeutic (lavage) indication with a 2.7 mm arthroscope (Storz, Tullingen, Germany). Eight samples were obtained from the suprapatellar pouch and the medial and lateral gutter in each patient. Four samples were fixed in 4 % formaldehyde and embedded in paraffin wax for immunohistochemistry and the remaining four collected on RLT lysis buffer (Qiagen, Crawley, UK) for RNA extraction. The present study was approved by the institutional ethics committee of Xi'an Jiao Tong University.

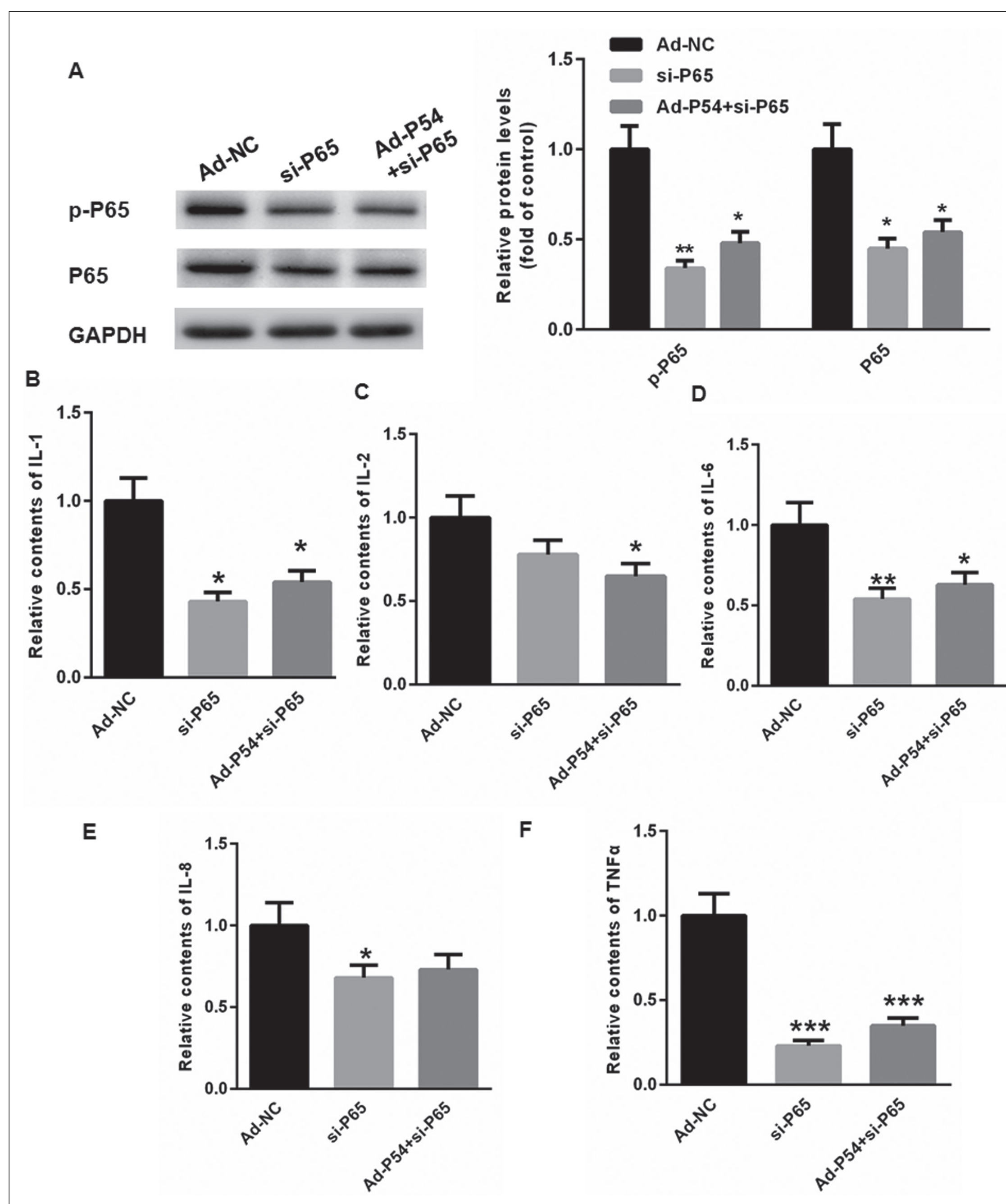

Fig. 5: p54 mediated pro-inflammatory responses mainly through NF-κB signaling. (A) Western blot analysis. ELISA analysis of IL-1 (B), IL-2 (C), IL-6 (D), IL-8 (E) and TNFα (F) levels. *P<0.05, **P<0.01.

4.2. Cell culture

SW982 cells were obtained from ATCC (Manassas, VA, USA) and grown in Dulbecco's modified Eagle's medium (DMEM; Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10 % fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA, USA) until confluent. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

4.3. Quantitative analysis of inflammatory cytokines

Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-8, IL-12, TNFα, and MCP-1 were purchased from Peprotech (Rocky Hill, NJ, USA) and run according to the manufacturer's instructions. Samples were run in duplicate, and each experiment was repeated independently at least three times.

4.4. Western blotting

Total proteins were isolated from tissues using a total protein extraction kit (Keygen, Nanjing, China). A total of 20 µg protein was separated using SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked with 5% fat-free milk at room temperature for 2 h. The immune-blot was incubated with primary antibody detecting p-P65, P65, P54 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1000 dilution; Santa Cruz Biotechnology) was used as a control. The signals were detected using a Super ECL Plus Kit (Keygen) and determined by quantitative analysis using UVP software (UVP, LLC, Upland, CA, United States).

4.5. Construction of adenovirus vectors

And the adenovirus vectors overexpressing and inhibiting P54 or NF-κB were constructed by Genechem (Shanghai, China).

4.6. Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using the Chromatin Immunoprecipitation Assay Kit (Millipore, Boston, Massachusetts, USA). Briefly, the nuclei DNA was extracted from cells and sonicated into 200-1000 bp. Precleared chromatin was immunoprecipitated with anti-USF1 and normal IgG antibodies according to the manufacturer's instructions. Immunocomplexes were added into 50 µl of protein A/G-Sepharose beads and purified with Qiaquick (QIAGEN, Düsseldorf, German) PCR purification columns. The precipitated DNA was amplified with p65-specific primers.

4.7. Promoter reporter analysis

The promoter region of p65 was amplified from the genomic DNA of SW982 cells. The PGL3 promoter vector and the amplified fragments were digested with *Xho*I/ KpnI and purified by gel electrophoresis. The digested fragment was then inserted into the PGL3 vector up-stream of the SV40 promoter. HEK293T cells were co-transfected with the PGL3 plasmids and the PRL-TK vector using the VigoFect Transfection Reagent (Beijing, China). The cells were harvested and lysed 48 h post-transfection. The relative light units (RLU) were determined using the Dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) according to the standard protocols. Normalized luciferase data (firefly/renilla) was compared with the empty pGL3-promoter vector. The RLU were determined using the dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA) according to the standard protocol.

4.8. Statistical analysis

Data were presented as mean±SD from 3 independent experiments or 5 mice. Statistical analysis was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared.

References

Basu A, Dong B, Krainer AR, Howe CC (1997) The intracisternal A-particle proximal enhancer-binding protein activates transcription and is identical to the RNA- and DNA-binding protein p54nrb/NonO. Mol Cell Biol 17: 677-686.
Cooles FA, Isaacs JD (2011) Pathophysiology of rheumatoid arthritis. Curr Opin Rheumatol 23: 233-240.
Costa AP, Lopes MW, Rieger DK, Barbosa SG, Goncalves FM, Xikota JC, Walz R, Leal RB (2016) Differential activation of mitogen-activated protein kinases, ERK 1/2, p38(MAPK) and JNK p54/p46 during postnatal development of rat hippocampus. Neurochem Res 41: 1160-1169.
Culshaw S, McInnes IB, Liew FY (2011) What can the periodontal community learn from the pathophysiology of rheumatoid arthritis? J Clin Periodontol 38 Suppl 11: 106-113.
Firestein GS (2003) Evolving concepts of rheumatoid arthritis. Nature 423: 356-361.
Gerber HP, Seipel K, Georgiev O, Hofferer M, Hug M, Rusconi S, Schaffner W (1994) Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science 263: 808-811.
Hinoi E, Yoneda Y (2011) Possible involvement of glutamatergic signaling machineries in pathophysiology of rheumatoid arthritis. J Pharmacol Sci 116: 248-256.
Ivashkiv LB, Hu X (2003) The JAK/STAT pathway in rheumatoid arthritis: pathogenic or protective? Arthritis Rheum 48: 2092-2096.
Keil JM, Liu X, Antonetti DA (2013) Glucocorticoid induction of occludin expression and endothelial barrier requires transcription factor p54 NONO. Invest Ophthalmol Vis Sci 54: 4007-4015.
Koning F (2015) Recent insight in the pathophysiology of coeliac disease: relevance to rheumatoid arthritis. Clin Exp Rheumatol 33: S8-10.
Malemud CJ (2013) Intracellular signaling pathways in rheumatoid arthritis. J Clin Cell Immunol 4: 160.
Mori T, Miyamoto T, Yoshida H, Asakawa M, Kawasumi M, Kobayashi T, Morioka H, Chiba K, Toyama Y, Yoshimura A (2011) IL-1beta and TNFalpha-initiated IL-6-STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis. Int Immunol 23: 701-712.
Mu N, Gu J, Huang T, Zhang C, Shu Z, Li M, Hao Q, Li W, Zhang W, Zhao J, Zhang Y, Huang L, WangS, Jin X, Xue X, Zhang W, Zhang Y (2016) A novel NF-kappaB/ YY1/microRNA-10a regulatory circuit in fibroblast-like synoviocytes regulates inflammation in rheumatoid arthritis. Sci Rep 6: 20059.
Noort AR, Tak PP, Tas SW (2015) Non-canonical NF-kappaB signaling in rheumatoid arthritis: Dr Jekyll and Mr Hyde? Arthritis Res Ther 17: 15.
Noort AR, van Zoest KP, van Baarsen LG, Maracle CX, Helder B, Papazian N, Romera-Hernandez M, Tak PP, Cupedo T, Tas SW (2015) Tertiary lymphoid structures in rheumatoid arthritis: NF-kappaB-inducing kinase-positive endothelial cells as central players. Am J Pathol 185: 1935-1943.
Ortiz MA, Diaz-Torne C, Hernandez MV, Reina D, de la Fuente D, Castellvi I, Moya P, Ruiz JM, Corominas H, Zamora C, Canto E, Sanmarti R, Juarez C, Vidal S (2015) IL-6 blockade reverses the abnormal STAT activation of peripheral blood leukocytes from rheumatoid arthritis patients. Clin Immunol 158: 174-182.
Park Y, Lee JM, Hwang MY, Son GH, Geum D (2013) NonO binds to the CpG island of oct4 promoter and functions as a transcriptional activator of oct4 gene expression. Mol Cells 35: 61-69.
Passon DM, Lee M, Rackham O, Stanley WA, Sadowska A, Filipovska A, Fox AH, Bond CS (2012) Structure of the heterodimer of human NONO and paraspeckle protein component 1 and analysis of its role in subnuclear body formation. Proc Natl Acad Sci USA 109: 4846-4850.
Pincus T, Sokka T, Wolfe F (2001) Premature mortality in patients with rheumatoid arthritis: evolving concepts. Arthritis Rheum 44: 1234-1236.
Pruijn GJ (2015) Citrullination and carbamylation in the pathophysiology of rheumatoid arthritis. Front Immunol 6: 192.
Raptopoulou A, Sidiropoulos P, Katsouraki M, Boumpas DT (2007) Anti-citrulline antibodies in the diagnosis and prognosis of rheumatoid arthritis: evolving concepts. Crit Rev Clin Lab Sci 44: 339-363.
Schiffner S, Zimara N, Schmid R, Bosserhoff AK (2011) p54nrb is a new regulator of progression of malignant melanoma. Carcinogenesis 32: 1176-1182.
Spurlock CF, 3rd, Tossberg JT, Olsen NJ, Aune TM (2015) Cutting edge: chronic NF-kappaB activation in CD4+ T cells in rheumatoid arthritis is genetically determined by HLA risk alleles. J Immunol 195: 791-795.
Swierkot J, Nowak B, Czarny A, Zaczynska E, Sokolik R, Madej M, Korman L, Sebastian A, Wojtala P, Lubinski L, Wiland P (2016) The activity of JAK/STAT and NF-kappaB in patients with rheumatoid arthritis. Adv Clin Exp Med 25: 709-717.
Totoson P, Maguin-Gate K, Prati C, Wendling D, Demougeot C (2014) Mechanisms of endothelial dysfunction in rheumatoid arthritis: lessons from animal studies. Arthritis Res Ther 16: 202.
Toussirot E, Michel F, Binda D, Dumoulin G (2015) The role of leptin in the pathophysiology of rheumatoid arthritis. Life Sci 140: 29-36.
Verma IM (2004) Nuclear factor (NF)-kappaB proteins: therapeutic targets. Ann Rheum Dis 63 Suppl 2: ii57-ii61.
Yin G, Wang Y, Cen XM, Yang M, Liang Y, Xie QB (2015) Lipid peroxidation-mediated inflammation promotes cell apoptosis through activation of NF-kappaB pathway in rheumatoid arthritis synovial cells. Mediators Inflamm 2015: 460310.
Zhai Y, Wu B, Li J, Yao XY, Zhu P, Chen ZM (2016) CD147 promotes IKK/IkappaB/ NF-kappaB pathway to resist TNF-induced apoptosis in rheumatoid arthritis synovial fibroblasts. J Mol Med (Berl) 94: 71-82.
Zhang L, Luo J, Wen H, Zhang T, Zuo X, Li X (2016) MDM2 promotes rheumatoid arthritis via activation of MAPK and NF-kappaB. Int Immunopharmacol 30: 69-73.
Zhu Z, Zhao X, Zhao L, Yang H, Liu L, Li J, Wu J, Yang F, Huang G, Liu J (2016) p54(nrb)/NONO regulates lipid metabolism and breast cancer growth through SREBP-1A. Oncogene 35: 1399-1410.