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CCL3 participates in the development of rheumatoid arthritis by activating AKT

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Abstract. – OBJECTIVE: To investigate whether CC chemokine 3 (CCL3) could exert a certain effect on rheumatoid arthritis (RA) by regulating inflammatory responses and provide a new direction for the treatment of RA.

PATIENTS AND METHODS: Totally 47 RA patients (10 males and 37 females) with complete clinical data were included. Meanwhile, 27 healthy volunteers with same age and gender were recruited as healthy controls. The mRNA and protein level of CCL3 in the peripheral blood mononuclear cells (PBMCs) of RA patients and normal controls were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. The inflammatory infiltration of synovial tissue was observed by hematoxylin and eosin (HE) staining. Immune fluorescence was used to further analyze the level of CCL3 in T and B cells of synovial tissue in RA patients. Simultaneously, real-time flow cytometry was applied to detect the level of CCL3 in T and B cells of PBMCs in the normal control group and the RA group. Western blot was used to detect the level of pAKT in RA-FLS treated with different concentrations of recombinant human CCL3. Besides, enzyme-linked immunosorbent assay (ELISA) was applied to detect the levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and receptor activator of nuclear factor kappa-B ligand (RANKL) in the culture supernatant of RA-FLS stimulated by different doses of recombinant human CCL3.

RESULTS: The level of CCL3 in peripheral blood and synovial fluid of RA patients was markedly higher than that of normal controls. Inflammatory cells were infiltrated in synovial tissue of RA patients. Meanwhile, CCL3 was mainly expressed in CD4+ T cells. CCL3 treatment in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) could activate the PI3K/AKT signaling pathway to different degrees and increase the expression of cytokines including interleukin-6 (IL-6), IL-1β, TNF-α, and RANKL. These results indicated that CCL3 might participate in the progression of RA by activating AKT.

CONCLUSIONS: We showed that CCL3 enhanced the expression level of pro-inflammatory cytokines such as IL-6, IL-1β, TNF-α, and RANKL by activating the PI3K/AKT signaling pathway. Besides, CCL3 could up-regulate CD4+T cells to mediate the inflammatory response of RA. These findings might provide new directions for the prevention of RA.

Key Words:
CCL3, RA, Inflammation, PI3K/AKT.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease with the main clinical manifestation of arthritis[1]. The basic pathological features of RA are synovitis and vasculitis. Synovial vascular may form vasospasm due to hyperplasia within the joint, resulting in thickening of synovial membrane as well as increased exudation and secretion of a variety of cytokines. These may eventually lead to cartilage violation and bone damage[2-4]. The etiology and pathogenesis of RA have not been fully explained[4-6]. Studies have shown that the abnormal activation of T cells, especially CD4+ T cells, and secretion of cytokines can participate in the excitation and continuation of RA.

Chemokine is an alkaline secretory protein A superfamily with small molecular-weight. It enables chemotaxis of cells, participates in the recruitment and activation of various cells, and induces cell motility and degranulation[7-9]. In addition, chemokine also plays an important role in the development of various immune cells and organs, immune responses, and inflammatory reactions. According to the relative position and number of N-terminal cysteine residues, chemokines can be divided into four subfamilies, including C, CC, CXC, and CX3C[10,11]. CC

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chemokine 3 (CCL3), also known as macrophage inflammatory protein 1-a, is a member of the CC subfamily. It's known that CCL3 is produced by monocytes/macrophages, lymphocytes, neutrophils as well as immune cells such as basophils, mast cells, fibroblasts, and dendritic cells. Meanwhile, CCL3 exerts various biological effects by binding to its three cell surface receptors, including CCR1, CCR3, and CCR5[12,13]. MIP-1a induces a variety of pro-inflammatory activities such as leukocyte chemotaxis, and promotes the entry of T cells into the inflammatory tissue region from blood circulation. Chemotactic CD4+ cells, CD8+ cells, natural killer cells, and dendritic cells bind to the corresponding receptors and coordinate the occurrence of immune reactions in the immune response site by migrating through vascular endothelial cells[14]. In addition, MIP-1a is considered as a key inflammatory mediator in granuloma, asthma, T1D as well as other autoimmune diseases[15,16].

Several studies have shown that CCL3 is closely related to the occurrence and development of inflammation. In this study, we aimed to explore the expression of CCL3 and its related inflammatory cytokines in RA patients and cell lines. In addition, we hoped to explore the possible role of CCL3 in RA and related signal pathways to provide new ideas and directions for the prevention and treatment of RA.

Patients and Methods

Research Subjects

Totally 47 RA patients including 10 males and 37 females who received treatment were enrolled in this study. Complete clinical data of these subjects were obtained. This study was approved by the Ethics Committee of the Affiliated Hospital of Taishan Medical University. Signed written informed consents were obtained from all participants before the study. The age of these patients ranged from 19 to 79, with an average age of (53±16) years old. Synovial tissues were obtained from 10 RA patients who received knee arthroplasty. At the same time, 27 healthy and age-matched volunteers were recruited as healthy controls, including 22 females and 5 males. The age of these healthy controls was from 16 to 75, with an average age of (52±18) years old. CCL3-mRNA of PBMCs test was performed in all healthy controls and RA patients. Meanwhile, 8 healthy controls and 9 RA patients were selected for peripheral blood flow cytometry. Immunohistochemically and immunofluorescent staining were performed in the synovial tissues of 10 RA patients and 5 healthy controls.

Immunohistochemistry

The synovial tissue was immediately removed, fixed in 10% formaldehyde, and embedded in paraffin. After dehydration with xylene and series of ethanol, the slices were incubated with 3% H_2O_2 for 10 min. After that, the slices were placed in an antigen-recovery solution (sodium persulfate buffer in pH 6.0) for high-pressure heat recovery. After blocking 15 minutes with 3% bovine serum albumin, the slices were incubated with CCL3 antibody (1:100 dilution, rabbit anti-human, Abcam, Cambridge, MA, USA) at 4°C overnight. On the next day, the slices were washed with phosphate-buffered saline (PBS), followed by incubation with immunohistochemically universal secondary antibody (ZhongshanJinqiao, PV-6000, Beijing, China) at 37°C for 30 min. Subsequently, the slices were washed with PBS and stained with diaminobenzidine (DAB) under a microscope (Beyotime, Shanghai, China). After DAB staining, the slices were counterstained with hematoxylin, washed with water, decolorized with l% hydrochloric acid in ethanol, and placed back in water. After dehydration in a series of ethanol and xylene, the slices were sealed with neutral resin for further counting.

Immunofluorescence

To localize CCL3-positive cells, T and B cells were labeled with CD4 antibody (mouse anti-human, Jinshan Zhongqiao, China) and CD20 antibody (mouse anti-human, ZhongshanJinqiao, Beijing, China) double-fluorescently, respectively. Conventional immune-histochemical method was applied for specimen fixation and treatment. Then, the slices were incubated with CCL3/CD4 antibody or CCL3/CD20 antibody at 4°C overnight. On the next day, the slices were incubated with goat anti-rabbit and goat anti-mouse secondary antibodies (Jinqiao, Shanghai, China) at 37°C in the dark for 30 min. Finally, the slices were stained with 4',6-dimeryl-2, DAPI (4',6-diamidino-2-phenylindole, Thermo Fisher Scientific, Waltham, MA, USA) for further observation under a fluorescence microscope.

Western Blot Analysis

Peripheral blood mononuclear cells were collected, and radio-immunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) was employed to extract total protein of cells. Ex-

tracted proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) at 80 V for 60 min in an ice bath. After blocking with 5% skim milk at room temperature for 2 h, the membranes were incubated with primary antibodies (CCL3, β-actin, and GAPDH in 1:1000 dilution) at 4°C overnight. After washing three times with Tris-Buffered Saline with Tween 20 (TBST 20; Beyotime, Shanghai, China), the membranes were incubated with corresponding secondary antibody (horseradish peroxidase labeled IgG antibody 1:10000) at room temperature for 1-2 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Real-Time Fluorescence Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells of each group according to the manufacturer's protocol of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and quantitative analysis were performed based on the instructions of PrimerScript RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan). Relative expression level of genes was calculated by the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated 3 times. Primers used in this study were shown in Table I.

PBMCs Extraction

A total of 2 mL peripheral blood samples from RA patients and healthy controls were placed in EDTA-Na anticoagulant tubes and mixed gently, followed by centrifugation with gradient density. The remaining portion of the tube was mixed with an equal volume of phosphate-buffered saline (PBS), and placed into the erythrocyte lysate. After washing with PBS buffer twice, the pellets were re-suspended in 100 μL PBS to obtain PBMCs suspension.

Flow Cytometry

PBMCs were made into cell suspensions. First, the cells were incubated with CCL3 primary antibody and secondary antibody for 40 min. After washing with PBS 3 times, CD8-FITC, CD4-polypyridoxanthin-chlorophyll-protein complex (PerCP), CD20, and all phycocyanin (APC) were added to the cells at 4°C in the dark for 30 min. Flow cytometry was performed on BD FACSCalibur, InfluxTM. All fluorescent-labeled antibodies were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). FlowJo 7.6.1 was used for imaging and data analysis.

Cytokine Level Determination

The culture supernatant was collected after RA-FLS. The optical density (OD) values of each well at the wavelengths of A562 nm and A450 nm were measured with a microplate reader according to the instructions of the enzyme-linked immunosorbent assay (ELISA) kit (Bio Legend, San Diego, CA, USA). Finally, the cytokine concentration was calculated according to the standard curve of absorbance value.

Statistical Analysis

Graphpad Prism (v6.0, La Jolla, CA, USA) was used for all statistical analysis. Data were expressed as mean ± standard error. The *t*-test was used to compare the differences between the two groups. Measured data with non-normal distribution were expressed as median (25^{th} percentile, 75^{th} percenti-

Table I. Expression of CCL3 in peripheral blood T cells from patients with RA and healthy controls.

Number of cases		Proportion of CCL3-positive cells (%)			Average fluorescence intensity of CCL3 expression		
		CD4+T cell	CD8+T cell	CD20+B cell	CD4+T cell	CD8+T cell	CD20+B cell
NormalGroup	8	0.64 (0.60, 0.68)	0.54 (0.53, 0.56)	0.32 (0.29, 0.36)	119 (81, 173)	94 (74, 122)	52 (33, 105)
RA Group	9	0.99 (0.94, 1.00)	0.77 (0.73, 0.79)	0.34 (0.30, 0.38)	158 (117, 246)	99 (74, 119)	63 (34, 81)
U		25	24	26	36	36	35
p-value		0.009*	0.062	0.370	0.024*	0.842	0.901

[M (P_{25}, P_{75})] *$p<0.05$

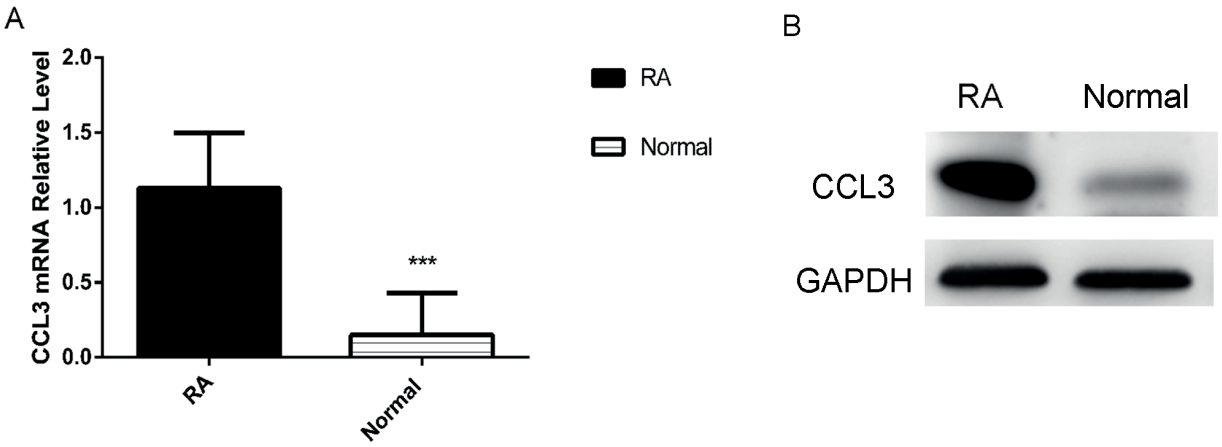

Figure 1. CCL3 was involved in the inflammation of RA. ***A,*** The mRNA expression of CCL3 in the peripheral blood mononuclear cells of RA patients and healthy controls was detected by qRT-PCR. Each group had 20 specimens, ***$p<0.001$. ***B,*** The protein expression of CCL3 in the peripheral blood mononuclear cells of RA patients and healthy controls was detected by western blot. Each experiment was repeated for 3 times.

le) [M (P25, P75)] using the Mann-Whitney U test. Correlation analysis was performed according to the Pearson correlation analysis. All experiments were repeated at least three times. $p<0.05$ was considered statistically significant.

Results

CCL3 Participated in the Inflammation of RA

To investigate whether CCL3 was involved in the development of RA, Western blot and qRT-PCR were performed to detect the protein and mRNA expression of CCL3 in the peripheral blood (Figure 1A, 1B) of RA patients and healthy controls, respectively. Results showed that both the protein and mRNA expression levels of CCL3 were strikingly increased in the peripheral blood of RA patients. At the same time, the level of CCL3 in synovial tissue of RA patients was detected by immunohistochemistry. The results indicated that there was no positive expression of CCL3 in the synovial tissue of healthy controls (Figure 2A). However, a large number of inflam-

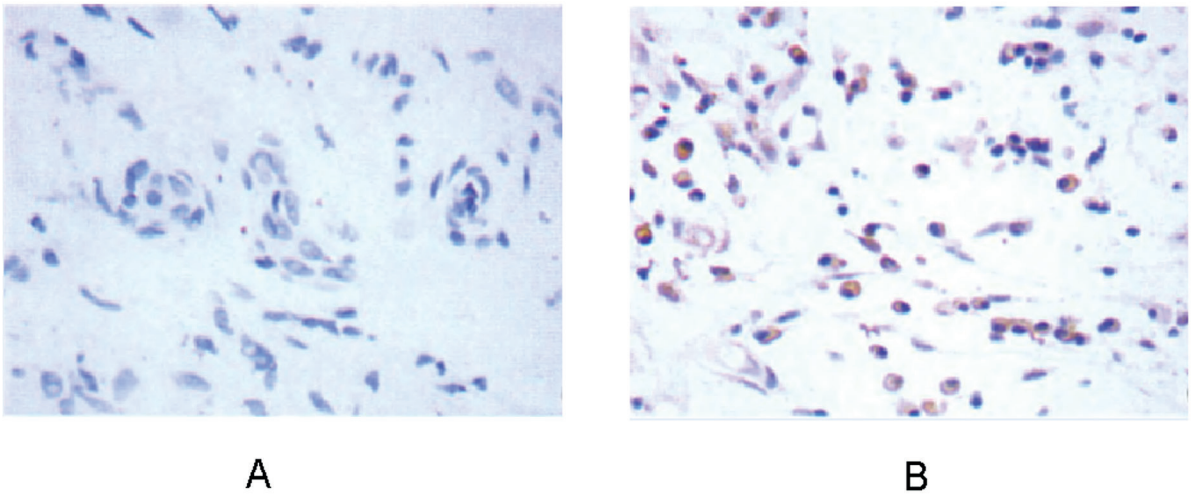

Figure 2. CCL3 was up-regulated in the inflammation of RA. The expression of CCL3 in the synovial tissue of RA patients detected by immunohistochemistry was amplified 400 times under a light microscope. ***A***: No CCL3 positive expression was observed in the synovial tissue of healthy controls; ***B***: A large number of inflammatory cell infiltration and CCL3 expression were observed in the synovial tissue of RA patients.

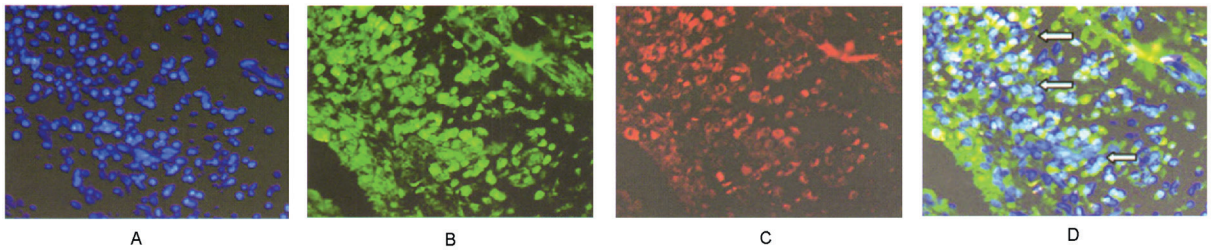

Figure 3. CCL3 enhanced the inflammatory response of RA by modulating CD4+ T cells. Immunofluorescence was used to further analyze the localization of CCL3 in synovial T and B cells as well as the expression of CCL3 in CD4+ T cells of synovial tissue. Immunofluorescence double staining x400. ***A***, Nucleus for 4',6-dimercapto-2-phenyl hydrazine staining (blue); ***B***, positive CCL3 expression (green); ***C***, CD4+ T cells (red); ***D***, CD4+ T cells simultaneous expressing CCL3 (indicated by arrows, red fluorescence and green fluorescence were yellow after superimposed).

matory cell infiltration was observed in the synovial cells of RA patients with high expression of CCL3 in these inflammatory cells (Figure 2B). In addition, the mRNA expression level of CCL3 in the synovial fluid mononuclear cells of the knee joint were detected in 13 RA patients (0.68±0.37) and matched PBMCs (0.66±0.56). We found that there was a significant correlation between the two groups (r=0.905, p=0.008). These results demonstrated that CCL3 was essential in the synovial fluid of RA and might participate in its inflammatory response.

CCL3 Enhanced the Inflammatory Response of RA by Regulating CD4+ T Cells

To further explore the role of CCL3 in RA inflammatory response, we analyzed the localization of CCL3 in synovium cells by immunofluorescence techniques. Results showed that CCL3 was mainly expressed in the cytoplasm of CD4+ T cells in the synovial tissue of RA patients (Figure 3), while CCL3 was positively expressed in CD20+ B cells (Figure 4). Meanwhile, flow cytometry was applied to detect PBMCs in 9 RA patients and 8 healthy controls for further investigating the expression of CCL3 in the peripheral blood lymphocyte subsets. Results indicated that CCL3 was highly expressed in CD4+ T cells, CD8+ T cells, and CD20+ B cells in RA peripheral blood. Statistically significant difference was only found in the CD4+ T cell group. The mean fluorescence intensity (MFI) of CCL3 expression in CD8+ T cells and CD20+ B cells was higher than that of healthy controls. The difference between the two groups was only statistically significant in the CD4+ T cell group (Table I). These above findings demonstrated that CCL3 could enhance the inflammatory response of RA by upregulating CD4+ T cells.

CCL3 Activated the Inflammatory Response of RA Via the Activation of the P13K/AKT Signaling Pathway

To further explore the mechanism of CCL3 involvement in RA inflammatory responses, we explored the activation of classical inflammation and transcriptional signaling pathways. RA-FLS was stimulated by 10, 50, 100, and 200 μg/L recombinant human CCL3, respectively. Subsequently, the

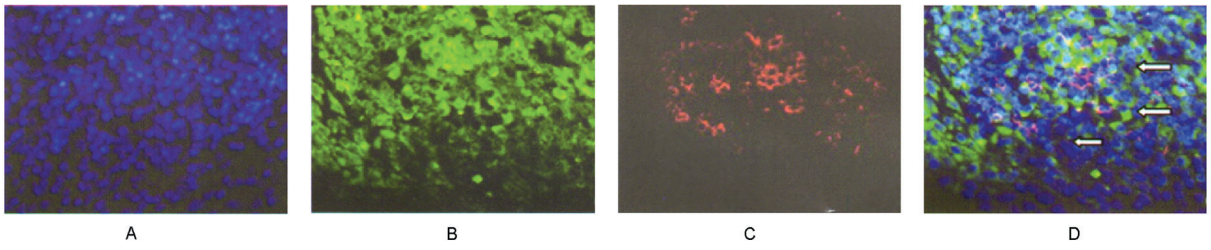

Figure 4. CD20+ B cell was not involved in CCL3 induced inflammatory response in RA. Expression of CCL3 in CD20 + B cells of synovial tissue. Immunofluorescence double staining x400. ***A***, Nucleus 4', 6. Dimercapto-2-phenylanthraquinone staining (blue); ***B,*** positive CCL3 expression (green); ***C***, CD20+ B cells (red); ***D***, CD20+ B cells with almost no expression of CCL3 (arrows indicated no obvious fluorescence superposition).

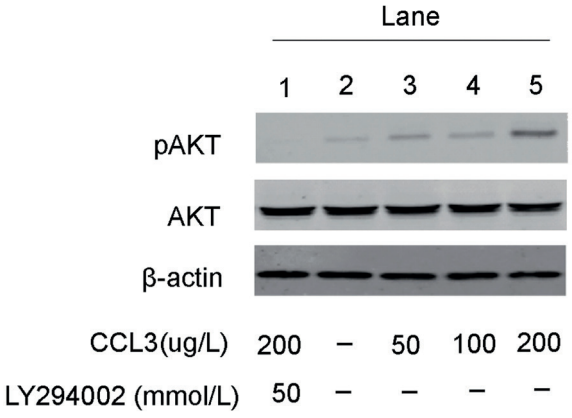

Figure 5. CCL3 activated the inflammatory response of RA by activating the P13K/AKT signaling pathway. The expression level of pAKT in RA-FLS after stimulation with different concentrations (10, 50, 100, 200 ug/L) of recombinant human CCL3 was detected by western blot. The experiment was repeated for 3 times in each group.

expression of p-AKT in RA-FLS stimulated with different concentrations of CCL3 was detected by Western blot. Results showed that the protein expression of p-AKT in RA-FLS after stimulation with CCL3 was markedly higher than that of the non-stimulated group (pAKT/AKT 0.24±0.09 *vs.* 0.14±0.06, t=2.62, p=0.02). After the addition of P13K inhibitor (LY294002, 50 mmol/L), 200 ug/L CCL3 administration could significantly stimulate the phosphorylation of AKT protein (Figure 5). This indicated that CCL3 could activate the PI3K/AKT signaling pathway. Next, ELISA was used to further verify the levels of interleukin-6 (IL-6), interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α) and receptor activator of nuclear factor kappa-B ligand (RANKL) in the culture supernatants after treated with different concentrations of recombinant human CCL3 in RA-FLS. We found that CCL3 significantly enhanced the levels of IL-6, IL-1β, TNF-α, and RANKL in a dose-dependent manner (p<0.05) (Figure 6). These data further illustrated that CCL3 could promote the secretion of cytokines and activate its inflammatory response by activating the PI3K/AKT signaling pathway in RA.

Discussion

RA is an autoimmune disease that mainly involves synovial joints. The pathological changes of RA mainly include chronic synovitis, vasospasm formation, bone destruction, and others[1,2]. Multiple cytokines are involved in its pathogenesis, and progressive joint destruction can occur as RA progresses. However, the etiology and pathogenesis of RA remain unclear[9,17,18].

Immunostaining and *in situ* hybridization have shown that the cells producing chemokines in RA are mainly synovial fibroblasts, macrophages, and VECs in affected joints[19]. The accumulation of chemokines in synovial fluid and synovial tissue during RA has been confirmed at both mRNA and protein levels. IL-8, growth factor related gene (Gro) and epithelial cell-derived neutrophil-activating factor (ENA-78) are wildly elevated. Meanwhile, CXC chemokines, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-la (MIP-la), MIP-1 B, RANTES that are mainly related to T cell and mononuclear cell infiltration are involved. CC chemokines are also abundantly expressed. Moreover, the expression of MIP-la and MCP-2 is induced with the progression of arthritis in type II CIA, indicating that inflammatory cell infiltration in RA is strongly correlated with chemokines produced in the inflammatory region[16,20].

In this work, we detected the levels of CCL3 in the peripheral blood of RA patients. Results implied that the expression of CCL3 in RA patients was strikingly increased when compared with healthy controls. Immunohistochemistry showed that a large number of inflammatory cell infiltration could be seen in the synovial tissue. Meanwhile, the expression of CCL3 in the peripheral blood and synovial fluid was extremely constant, indicating that CCL3 was involved in RA peripheral blood and synovial fluid, and might play a vital role in the inflammatory response of RA.

Studies have shown that IL-17 is produced by CD4+ T cells. Meanwhile, the level of IL-17 in the synovial tissue and peripheral blood of RA patients is elevated, which can up-regulate pro-inflammatory cytokines such as IL-6, TNF, and others, as well as promote the expression of chemokines and neutrophils to induce the migration of activated T cells[21-23]. To investigate the possible mechanism of CCL3 involvement in RA inflammatory response, immunofluorescence was applied to further analyze the localization of CCL3 and to clarify the expression of CCL3 in synovial T and B cells, as well as the type of CCL3 positive cells. Results showed that CCL3 could remarkably enhance the inflammatory response of RA by upregulating CD4+ T cells.

Previous researches have shown that fibroblast-like synoviocytes (FLS) exert a crucial ef-

fect on maintaining normal joint balance. FLA in RA is characterized by the loss of contact inhibition of anchorage-independent growth and abnormal proliferation. Meanwhile, FLA can secrete a variety of inflammatory cytokines and interact with locally infiltrated inflammatory cells, thereby participating in the process of RA joint destruction. Studies have reported that MIP-1a binds to CCR5 and dissociates the G protein heterologous trimer into α and βγ subunits. The α subunit inhibits adenylyl cyclase, and the βγ subunit activates phosphatidylinositol kinase (PI3K) and phospholipase C (PLC)[24].

Therefore, we stimulated RA-FLS with different concentrations of recombinant human CCL3, and found that the protein expression of p-AKT in the stimulation group was significantly higher than that of the non-stimulated group. However, the phosphorylation of AKT protein was significantly reduced after the addition of P13K inhibitor and CCL3 stimulation. The above results all suggested that CCL3 could activate the PI3K/AKT signaling pathway and promote cytokine secretion of RA to activate its inflammatory response.

We found that the level of CCL3 was higher in RA patients and could be involved in the inflammation of RA by upregulating CD4+ T cells. We further showed that CCL3 activated the PI3K/AKT signaling pathway and promoted the secretion of cytokines. Therefore, CCL3 might become a therapeutic intervention target for RA.

Conclusions

We observed that the CCL3 activated pro-inflammatory cytokines and RANKL by regulating the activation of the PI3K/AKT signaling pathway. Moreover, it upregulated CD4+ T cells to mediate the inflammatory response of RA. Our research might provide new insights and directions for the treatment of RA.

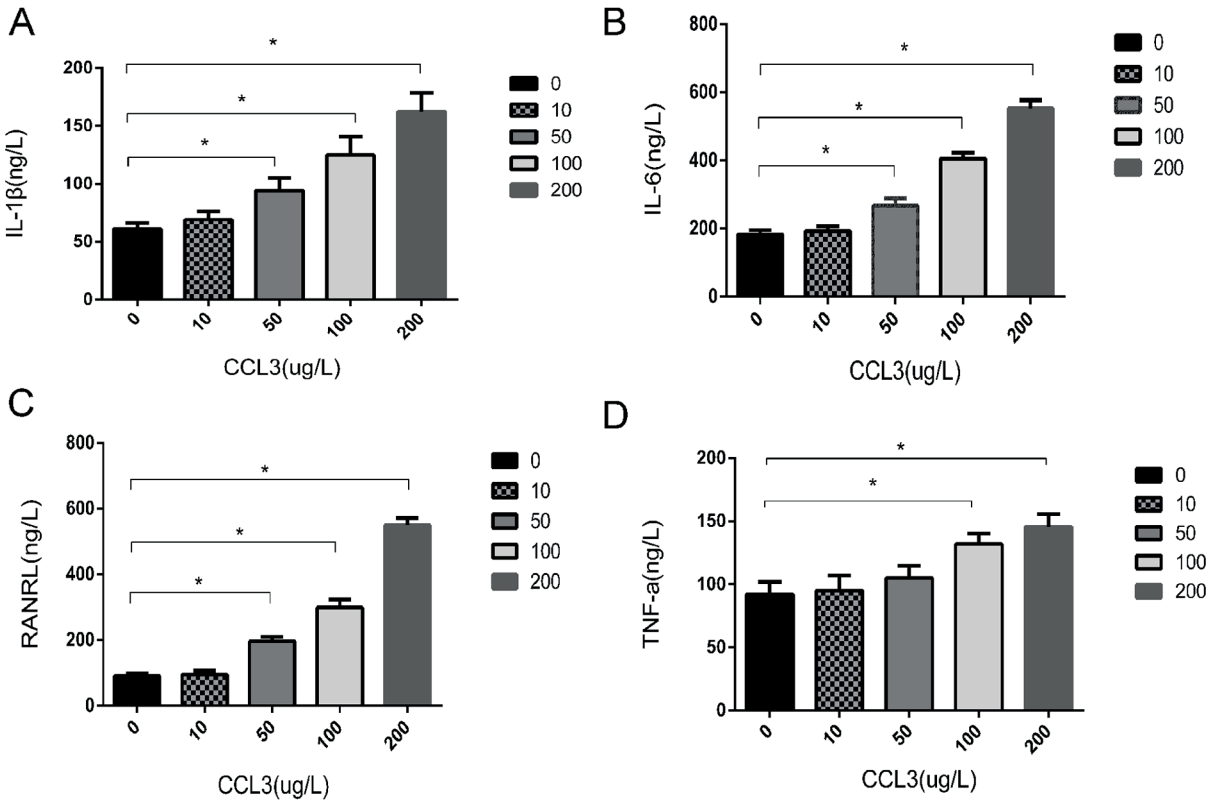

Figure 6. CCL3 activated inflammatory factors to participate in inflammatory response. The expression of IL-6(A), IL-1β(B), TNF-α(C) and RANKL(D) in the culture supernatants after RA-FLS stimulation with different concentrations (10, 50, 100, 200 ug/L) of recombinant human CCL3 was detected by ELISA. *p <0.05.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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