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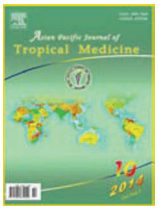

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Role of NLRP3 and NLRP1 inflammasomes signaling pathways in pathogenesis of rheumatoid arthritis

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ABSTRACT

Objective: To investigate the role of NLRP3 and NLRP1 inflammasomes signaling pathways in rheumatoid arthritis (RA). **Methods:** A total of 36 patients with RA were selected, peripheral blood mononuclear cell (PBMC) and granulocyte were separated from venous blood. RT-qPCR method was used to detect the expression level and diversity of NLRP3 and NLRP1 in PBMC and granulocyte mRNA in patients with RA, and detect the mRNA expression of downstream factor IL-1β. The correlation between RA and the expression of NLRP3 and NLRP1 was analyzed. Normal 30 cases were set as control group. **Results:** Expression levels of NLRP1, and caspase-1 mRNA in PBMC of RA group were significantly lower than those of control group (P<0.05), while there was no significant difference in expression levels of NLRP3, ASC, IL-1β mRNA between these two groups (P>0.05); NLRP3, caspase-1, and ASC mRNA expression in granulocyte of RA patients were significantly lower than those in control group (P<0.05). There was no correlation between rheumatoid factor and expression levels of NLRP3, ASC, caspase-1 mRNA in RA group (P>0.05); NLRP1, IL-1β mRNA expression level had a negative correlation with anti-rheumatoid factor antibody (P=0.0332, 0.0340). **Conclusions:** NLRP3 and NLRP1 inflammasomes signaling pathways are involved in RA inflammatory reaction process as protective factors, and play an important role in RA inflammatory mechanisms.

1. Introduction

Rheumatoid arthritis (RA) is a clinical common disease, which is a kind of autoimmune diseases mainly involving limb joints, with joint synovitis as the main clinical features. The incidence of RA is about 10% worldwide[1]. But RA pathogenesis is not yet fully understood, and there is a big difference between the predicting prognosis and the treatment of RA[2]. Most studies believed that RA is caused by genetic and environmental multi-factors, with innate and adaptive immunity involved in the process[3-7]. NLRP3, NLRP1 inflammasomes signaling pathways are the key receptors to initiate the innate and adaptive immunity, which promote the mature and release of pro-IL-1β in the form of IL-1β by binding to protein ASC, then binding to pro-caspase-1 and activating caspase-1, thus mediate inflammatory responses[8,9]. Clinically researches in NLRP3 and NLRP1 inflammasomes activation mainly focus on innate immune cells, which is rare in RA[10]. To survey the action mechanism of NLRP3 and NLRP1 inflammasomes signaling pathways in RA pathogenesis, the author detected expression levels and differences of NLRP3 and NLRP1 inflammasomes in peripheral blood mononuclear cell (PBMC) and granulocytes of selected RA patients using RT-qPCR.

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2. Materials and methods

2.1. Subjects

A total of 36 RA patients were selected (RA group), including 4 males, 32 females. The average age of thesse patients was (37.4±3.2) years (range, 22 to 56 years). All selected RA patients were conformed according to Revised Classification Criteria for Rheumatic Diseases of American Colloge of Rheumatology. Clinical manifestations of RA are as below: morning stiffness longer than 6 weeks for at least 1 h every day; at the same time with at least three joint areas of arthritis, hand joint swelling involved the wrist, metacarpophalangeal or proximal interphalangeal joints for more than 6 weeks; rheumatoid nodules with rheumatoid factor (RF) detected by sera diagnosis; X-ray changes indicating osteoporosis or bone erosion in affected joints. A total of 30 normal people after medical examination were selected as control group including 7 males, 23 females, aged from 17 to 58 years old, with an average age of (34.2±4.5) years. This group of patients had no family history of rheumatoid disease, laboratory examination without autoimmune diseases and infectious diseases. Before the start of the experiment, two groups of subjects agreed and signed the informed consent.

2.2. Agents and equipments

Real-time PCR amplification, enzyme standard instrument, PCR amplification, T2A gel imager (Bio-Rad company, USA); ultraviolet spectrophotometer, low speed centrifuge (Eppendorf, Germany); voltage steady flow PCR electrophoresis apparatus, PCR electrophoresis tank, dry type thermostat (Beijing LiuYi Instrument Factory); human peripheral blood lymphocyte separation medium (Tianjin Boyang Biological Technology Co., LTD); real-time eight league row tube, Trizol reagent, reverse transcription PCR kit (Bio-Rad company, USA); SYBR Green, agarose (Beijing Kangwei Century Biotechnology Co., LTD.); PCR primers, OSP kit (Shanghai Shenggong Biotechnology Engineering Company); Concentrated DAB kits (Beijing wipes Golden Bridge Biotechnology Co., LTD.); ASC, NLRP3, IL-10 antibody (Abeam Company) were purchased and prepared for the study.

2.3. Preparation of cDNA of PBMC

2.3.1. Separation of PBMC from lymphocyte separation medium

Three milliliters of venous blood were extracted from sterile tubes containing heparin, then an equal amount of Hanks was added to dilute blood. Two milliliters of lymphocyte separation medium were added to two 10 mL Ep pipes, 3 mL of diluted blood was slowly added along the pipe wall to make sure that the blood and liquid separation formed an obvious interface, then centrifuged at 2000 r/min for 20 min at room temperature. *In vitro* upper-middle-layer was transparent liquid, the middle layer was hoar peripheral blood mononuclear cells layer, lower layer was colorless lymph cell layer, the bottom layer was for the grain of red blood cells layer. A single cell layer was drawn to the new Ep tube with sterile dropper, 3 mL of Hanks liquid was added, centrifugated at 2000 r/min for 10 min. After removing the supernatant, 3 mL of Hanks liquid was added, and centrifugated at 2000 r/min for 10 min again. After removing the supernatant, 1 mL of Trizol liquid was added, stored at −80 °C.

2.3.2. RNA extraction from PBMC

RNA was extracted from PBMC by Trizol method. PBMC and granulocyte Ep tubes were thawed and dissolved at room temperature, 0.2 mL of chloroform was added, then the tube was shaked vigoursly for 15 seconds, incubated for 3 min at room temperature, and then centrifuged at 12000 r/min for 15 min at 4 °C. The upper layer was clear water phase (RNA), the middle layer was white (proteins and DNA), the bottom layer was chloroform phase. Then, 600 μL of upper clear water phase was extracted into a new Ep tube, added with 0.5 mL of opropyl alcohol, reversed for blending and incubated for 10 min at room temperature, centrifuged at 12000 r/min for 15 min at 4 °C; the white precipitation formed at the bottom of the tube edge was the RNA. Supernatant was abandoned, added with 1 mL of 75% ethanol. RNA precipitation was rinsed, centrifuged at 1500 r/min for 15 min at 4 °C, then supernatant was abandoned, the RNA precipitation was dried at room temperature for 5-10 min. RNA precipitation was dissolved with 20 μL of 0.1% diethylpyrocarbonate water followed by repeatedly percussion to accelerate the RNA precipitation dissolving, after 5 min at room temperature, concentration and purity of RNA sample were detected.

2.4. Preparation of cDNA of granulocyte

Three milliliters of venous blood from sterile tubes containing heparin were extracted, then added with 3 mL of Hanks to dilute blood. Two milliliters of lymphocyte separation medium were added into two 10 mL of Ep pipes, then slowly added with 3 mL diluted blood along the pipe wall, ensuring that the blood and liquid separation formed an obvious interface, then centrifuged at 2000 r/min for 20 min at room temperature. After removing the supernatant, 6 mL of red blood cells lysis buffer was added in red layer, percussed fully, then placed for 10 min at room temperature; then centrifugated for 10 min (2000 r/min). Supernatant was abandoned, 3 mL Hanks liquid was added to centrifuge at room temperature for 10 min (2000 r/min); then supernatant was abandoned, added with Trizol liquid 1 mL, then moved to −80 °C for cryopreservation. Granulocyte RNA was extracted using Trizol method following the above mentioned steps in 2.3.2.

Table 1
Expression of NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in PBMC of two groups.

Groups	*n*	NLRP3	NLRP1	ASC	Caspase-1	IL-1 β
RA group	36	1.040±1.130	0.940±0.620*	0.940±0.623	0.870±0.850*	1.250±1.000
Control group	30	1.340±1.680	1.410±1.020	1.290±0.810	1.340±1.680	2.280±2.210

*Note: Compared with control group $P<0.05$.

2.5. RT-PCR

The semi quantitative RT-PCR was used to detect the expression of NLRP3 and NLRP1 inflammasomes in PBMC of the two groups and for the relative quantitative analysis[11].

2.6. Statistical methods

The data were analyzed by GraphPad Prism 5.0 statistical software and expressed as mean±SD. *t*-test was used to compare the differences between groups, using Mann-Whitney test to analyze the mRNA expression between the two groups. $P<0.05$ was considered as significant difference.

3. Results

3.1. Expression of NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in PBMC of two groups

In the PBMC of two groups, there was no statistical difference of NLRP1, ASC and IL-1 β ($P>0.05$); expression of NLRP3 and caspase-1 in the RA group were significantly lower than that of control group ($P<0.05$), the results are shown in Table 1 and Figure 1.

3.2. Expression of NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in granulocytes of two groups

In the granulocyte of two groups, there was no statistical difference of NLRP1, IL-1 β ($P>0.05$); expression of NLRP3, ASC and caspase-1 in the observation group granulocyte were significantly lower than that of control group ($P<0.05$), the results are shown in Table 2 and Figure 2.

Table 2
Expression of NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in granulocytes of two groups.

Groups	*n*	NLRP3	NLRP1	ASC	Caspase-1	IL-1 β
RA group	36	0.76±0.79*	0.93±0.65	0.61±0.42*	0.53±0.30*	1.71±1.61
Control group	30	1.63±2.11	1.40±1.48	1.12±0.84	0.89±0.74	1.17±0.86

*Note: Compared with control group $P<0.05$.

3.3. Correlation of RF and expression level of NLRP3 and NLRP1 inflammatory factors

NLRP3, ASC, caspase-1 mRNA expression level had no correlation with RF in RA group ($P>0.05$); NLRP1, IL-1 β

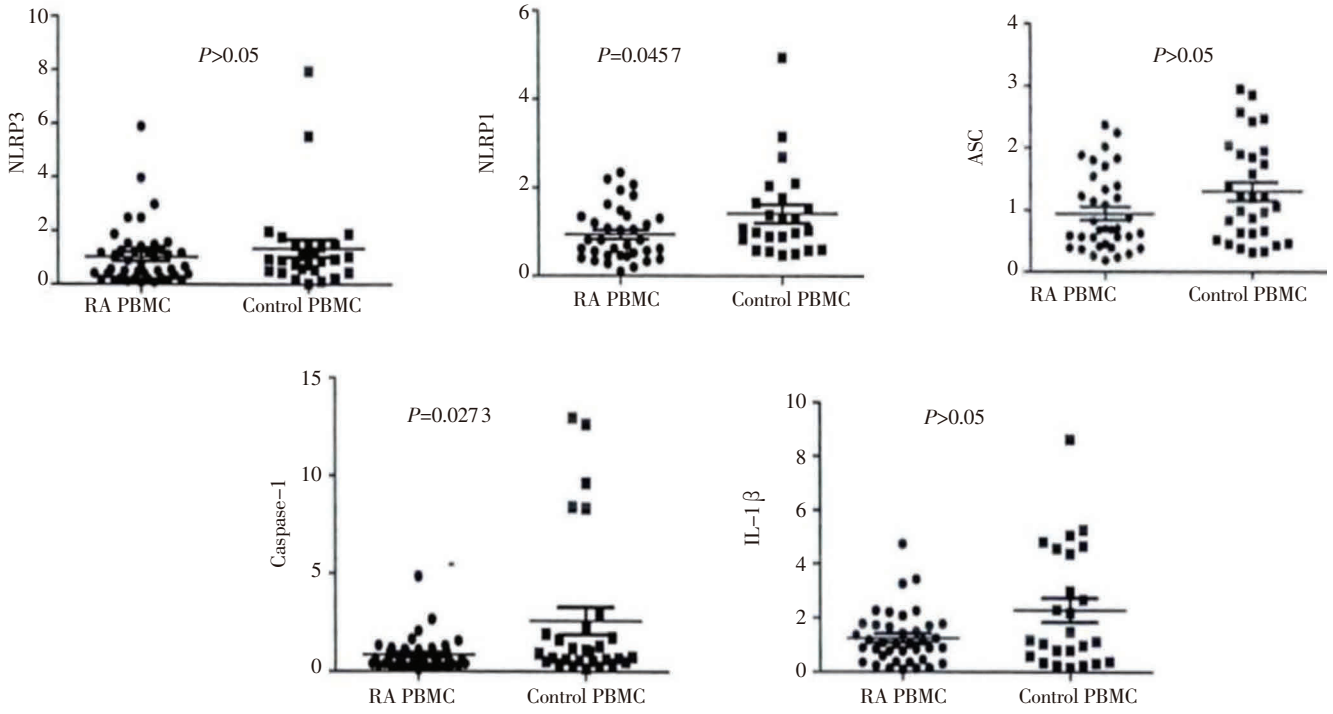

Figure 1. Expression of NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in PBMC of two groups.

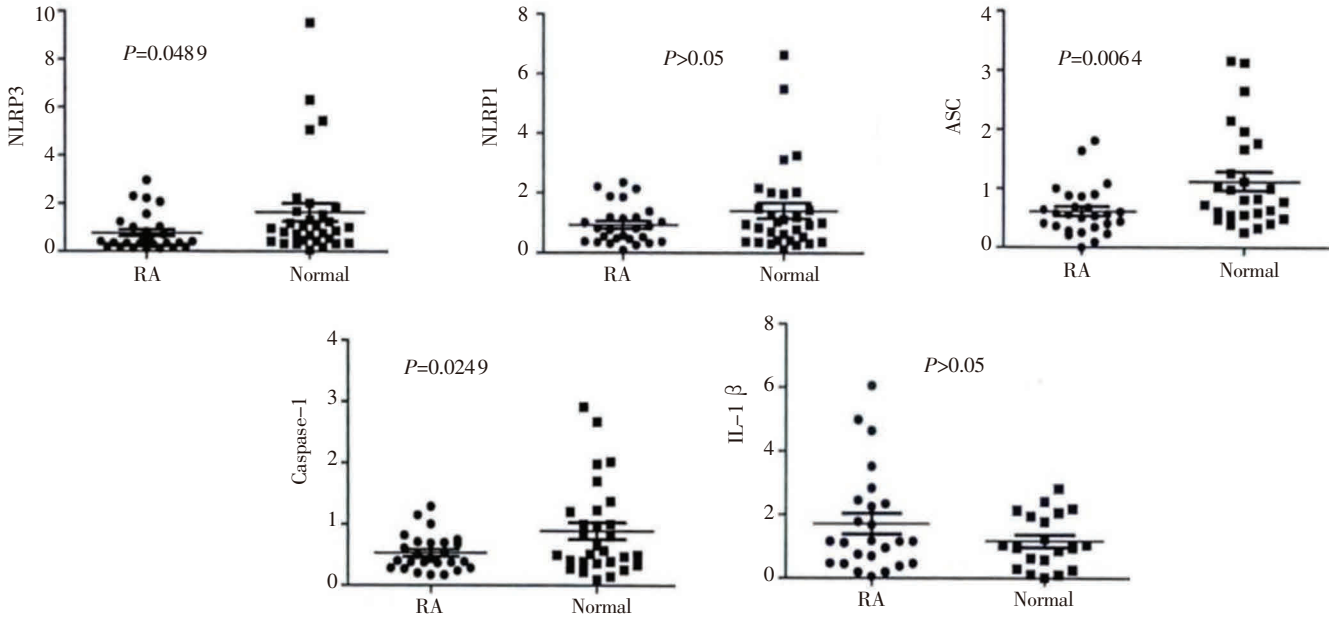

Figure 2. NLRP3, NLRP1, ASC, caspase-1 and IL-1 β in PBMC of two groups.

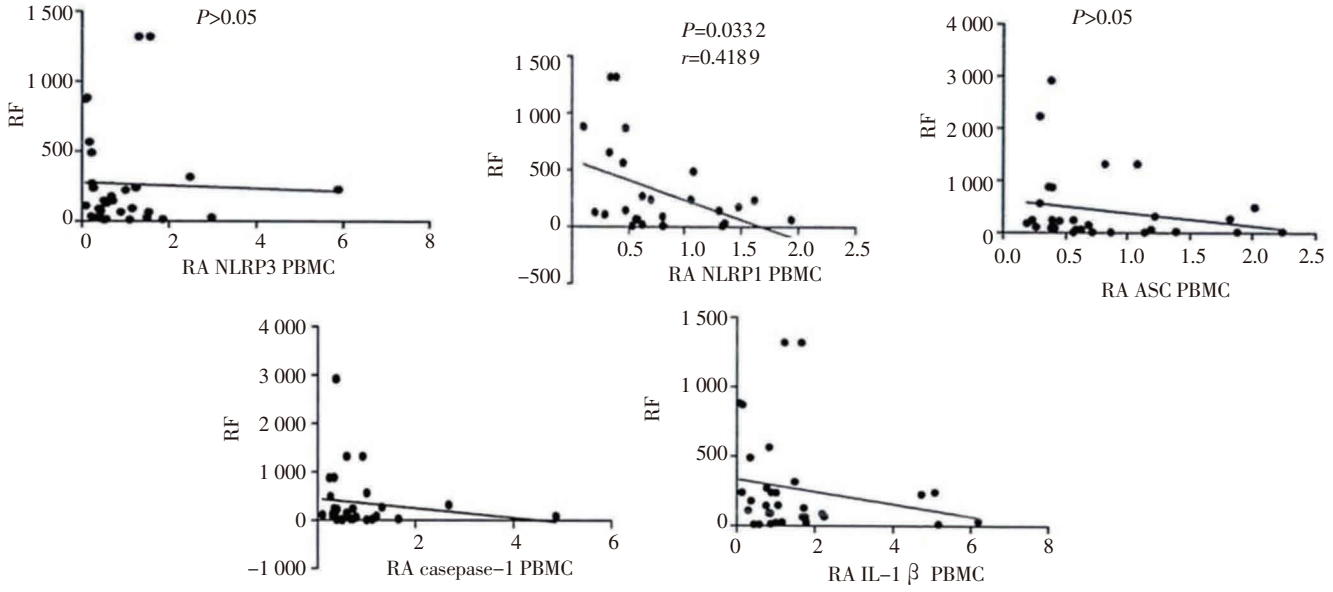

Figure 3. Correlation of RF and expression level of NLRP3 and NLRPl inflammatory factors.

mRNA expression level showed a negatively correlation with anti-RF antibody (P=0.0332 and 0.0340), as shown in Figure 3.

4. Discussion

RA is a clinically common autoimmune disease charterized by disabling multi-joint synovitis. The prevalence of RA is about 1% worldwide. The main pathological changes of RA is synovitis with the symptoms of a large number of line hairy on synovial surface bumped, inflammatory cells infiltration, the generation of new blood vessels, and thus pannus formed, and caused bone and cartilage tissue damage[11]. Clinical symptoms include articular dyskinesia, swelling pain, morning stiffness, *etc*. Late phrase of RA can affect the lung, heart, gastrointestinal tract, kidney, nervous system, blood system, and cause multiple organ damage[12]. As there are significant differences in duration, severity and prognosis of RA, if not treated earlier, the disease will gradually become worse. Therefore, early diagnosis and treatment of RA is of great significance to the prognosis of patients.

NLRP3 and NLRP1 are important members of NLR toll-like receptors, and important in the process of RA pathogenesis[13]. Some studies indicated that the disorder of NLRP3 and NLRP1 inflammasomes signaling pathways was associated with RA, gout, Crohn's disease, kidney inflammation and other autoimmune diseases[13,14]. Other scholars think that the abnormal up-regulation of inflammasomes expression level is the mechanism to induce autoimmune diseases[12]. A study showed that NALP3

expression was increased in gout mice[15]. Further research showed that the deletion of NALP3, ASC, caspase-1 can inhibit the mature and release of IL-1 β, and thus alleviate the inflammatory response in mice. Its mechanism may be related to the process of NALP3 recognition with binding to MSU, which enforces the inactive NALP3 inflammasome and its components activation to cause inflammatory reaction[16]. Other studies verified that the joint mutation of NLRP3 and CARD8 can lead to increased susceptibility of RA, which indicated that the polymorphism of NLRP3, NLRP1 inflammasomes signaling pathways is associated with RA susceptibility[10]. The results of this study showed that the expresion levels of NLRP1, caspase-1 mRNA of PBMC in RA patients were significant lower than that in the normal control group ($P<0.05$), and also the expression levels of NLRP3, ASC, caspase-1 mRNA of granulocyte were significantly lower than normal control group ($P<0.05$). It is suggesting that NLRP3, NLRP1 inflammasomes signaling pathways were involved in RA pathogenesis. One of the speculation is that a possible downgraded NLRP3, NLRP1 negative regulatory factors existing in RA inflammatory responses, which decreased the assembly of NLRP1 and NLRP3 inflammasomes and reduced the activation of caspase-1 via the NLRP3/1-signaling pathway. The other speculation is that NLRP3 and NLRP1 inflammasomes functioned as protective inflammatary factors involved in RA[10,15,17,18]. In this study, it showed a significant negative correlation between expression level of NLRP1 mRNA of PBMC and RF in RA patients, suggesting NLRP1 inflammasome signaling pathways involved in RA immune pathogenesis, and expression level of NLRP1 closely related to severity of RA.

According to the results of this study that NLRP3, NLRP1 inflammatory component expression lower in PBMC and granulocyte of RA patients, it shows that NLRP3, NLRP1 and NLRP3 inflammasomes signaling pathways involved in the process of RA inflammatory response as protective factors, and play an important role in RA inflammatory mechanisms.

Conflict of interest statement

We declare that we have no conflict of interest.

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