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Interleukin-6 (IL-6) Receptor Antagonist Protects Against Rheumatoid Arthritis

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The aim of this study was to investigate the protective effect of interleukin-6 (IL-6) receptor antagonist tocilizumab (TCZ) on rheumatoid arthritis (RA) and its related mechanism.





Material/Methods: Thirty RA patients receiving long-term methotrexate therapy at moderate and severe active stages were selected and treated with TCZ 8 mg/kg/time iv gtt intravenously guttae every 4 weeks. Peripheral blood was extracted before and 24 weeks after TCZ treatment. Peripheral blood mononuclear cells (PBMC) were collected by density gradient centrifugation. Flow cytometry was used to detect the ratio of CD4 naïve T cells and CD4 memory T cells, Th17 cells, and Treg cells in PBMC. DAS28 score, CRP, RF, and CCP levels in patients were evaluated.

Results: Compared with before treatment, IL-6 receptor antagonist TCZ significantly improved patients' condition, including DAS28 score, CRP, RF, and CCP levels ($P < 0.01$). Furthermore, TCZ obviously upregulated CD4 naïve T cells proportion and decreased CD4 memory T cells ratio ($P < 0.01$). TCZ also markedly reduced the proportion of Th17 cells and increased the proportion of Treg cells ($P < 0.01$).

Conclusions: TCZ can treat RA patients through regulating the ratio of CD4 naïve T cells, CD4 memory T cells, Th17 cells, and Treg cells in PBMC.

MeSH Keywords: **Antibodies, Bispecific • Antibodies, Monoclonal • Proton-Motive Force • Rheumatoid Factor**

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Background

Rheumatoid arthritis (RA) is a type of autoimmune disease characterized by chronic synovitis and bone erosion [1–3]. The pathogenesis of RA is still unclear. A variety of natural immune and acquired immune cells are involved in the RA process [4]. CD4 T cells abnormality has been confirmed to be the core cause of RA pathogenicity in human and mouse studies [5–7]. CD4 T cells are divided into naïve CD4 T cells and memory CD4 T cells. It was reported that the imbalance between naïve CD4 T cells and memory CD4 T cells plays a role in autoimmune disease [8]. Th17 cells and Treg cells were also found to have critical effects in RA. The proportion of Th17 cells is increased [9] and the proportion of Treg cells is decreased [10] in RA.

At first, researchers thought that reducing the number of lymphocytes and other immune cells or weakening their function could alleviate RA. This was confirmed by CD4 monoclonal antibody preliminary clinical trials, which also showed that it can slightly improve the condition of RA patients [11]. In addition, using CD20 monoclonal antibody to remove B cells can also improve RA patient condition [12].

Tocilizumab (TCZ), a humanized interleukin (IL)-6 receptor monoclonal antibody, is a recently developed biological agent that can effectively relieve RA disease [13]. An original study showed that IL-6 was a cytokine required for B cell differentiation [14]; therefore, TCZ can obviously change the proportion of B cells in peripheral blood in treating RA. However, IL-6 also plays an important role in T cell differentiation (Th1, Th2, and Th17) and Treg cell formation [15]. A recent study showed that TCZ can treat RA through mediating the Th1/Treg balance [15], but the mechanism by which TCZ works in RA treatment is not fully understood because of issues involving sample size, research cycle, and cell subset detection. Therefore, the present study used TCZ to treat RA patients and detected the ratio of CD4 naïve T cells and CD4 memory T cells, Th17 cells, and Treg cells in peripheral blood mononuclear cells (PBMC).

Material and Methods

Clinical subjects

Thirty RA patients receiving long-term methotrexate therapy at moderate and severe active stages were enrolled in this research at Tianjin People's Hospital of Nankai University. All the subjects were females, with a mean age at 35.53 ± 11.47 (25–48) years old. The BMI range was 20–25, and the RA course was 0.5–3 years. Methotrexate therapy was used for more than 3 months.

This study was pre-approved by the Ethics Committee of Tianjin People's Hospital of Nankai University. Written informed consent was obtained from all subjects.

Treatment method

Ten patients were treated by TCZ 8 mg/kg/time iv gtt intravenously guttae every 4 weeks.

PBMC extraction

Peripheral blood was extracted immediately before and at 24 weeks after TCZ treatment [16]. PBMC were collected by density gradient centrifugation. Peripheral blood was diluted by RPMI-1640 (Hyclone) at 1:1. Ten ml diluted peripheral blood was added to a 15-ml tube containing 5 ml lymphocyte separation medium (GE Healthcare) and centrifuged at 800 g for 18 min. After removing the white membrane layer, the liquid was moved to another 15-ml tube containing 10 ml serum-free RPMI-1640 medium and centrifuged at 600 g for 15 min. The cell sediment was resuspended in 10 ml serum-free RPMI-1640 medium and centrifuged at 400 g for 8 min. The collected cells were counted after trypan blue staining and used for the following experiment.

Peripheral blood CD4 naïve T cells and CD4 memory T cells ratio assay

PBMC was washed twice with PBS containing 5% BSA and centrifuged at 400 g for 8 min. After removing the supernatant, the cells were incubated in APC-CD4, PE-CD45 RA, and APC-CD45 RO at 4°C for 30 min in the dark. After washing twice with PBS containing 5% BSA and centrifuging at 400 g for 8 min, the cells were resuspended in 100 µl PBS for detection.

Th17 cell proportion detection

PBMC were pretreated by cell stimulus (Biolegend) for 6 h. The cells were washed twice with PBS containing 5% BSA and centrifuged at 400 g for 8 min. After removing the supernatant, the cells were incubated in APC-CD4 at 4°C in the dark for 30 min. Then the cells were incubated in 500 µl cell fixation and permeabilization liquid (BD) at room temperature for 30 min after removing the supernatant. After that, the cells were incubated in APC-IL-17 antibody at room temperature for 30 min after washing twice with permeabilization liquid (BD) and centrifugation. Finally, the cells were resuspended in 100 µl PBS for detection after removing the supernatant.

Treg cells proportion detection

After incubation in APC-CD4, the PBMC, separated as described above, were then incubated in APC-Foxp3 antibody

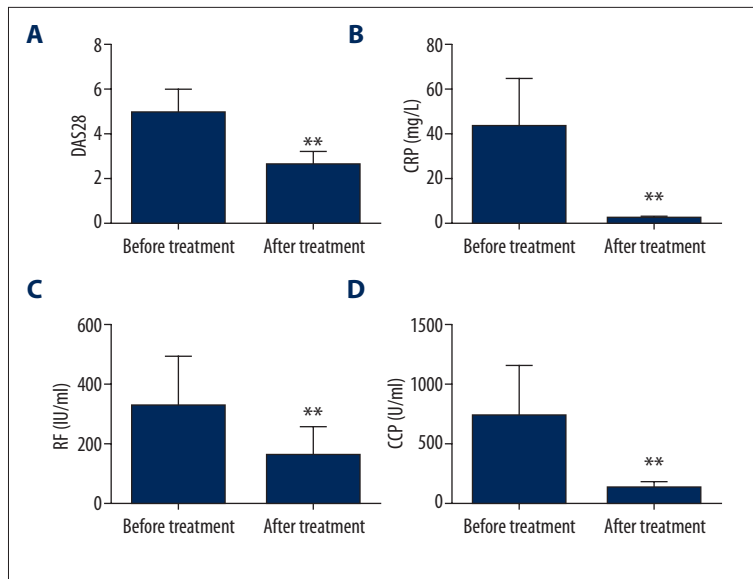


Figure 1. TCZ treatment impact on patient condition comparison. ** $P < 0.01$ (** compared with before treatment).

after removing the supernatant and were resuspended in 100 μ l PBS for detection.

Statistical analysis

SPSS16.0 software was used for data analysis. The *t* test was used for mean value comparison. $P < 0.05$ was considered as statistically significant.

Results

IL-6 receptor antagonist TCZ effect on patient condition

As shown in Figure 1, DAS28 score, CRP, RF, and CCP levels before treatment were 4.84 ± 1.23 , 42.80 ± 22.09 mg/L, 319.98 ± 172.63 U/ml, and 738.25 ± 437.41 U/ml, respectively. After TCZ treatment, DAS28 score, CRP, RF, and CCP levels were 2.61 ± 0.63 , 1.63 ± 0.94 mg/L, 154.40 ± 100.64 U/ml, and 135.85 ± 67.85 U/ml, respectively. Compared with before treatment, TCZ treatment significantly decreased DAS28 score, CRP, RF, and CCP levels ($P < 0.01$).

TCZ effect on CD4 naïve T cells and CD4 memory T cells ratio in peripheral blood

As shown in Figure 2, the proportion of CD4⁺CD45RA⁺T cells and CD4⁺CD45RO⁺T cells were $28.64 \pm 8.86\%$ and $68.93 \pm 14.64\%$ before treatment, respectively whereas they were $41.35 \pm 11.74\%$ and $41.85 \pm 10.35\%$ after TCZ therapy, respectively. TCZ obviously upregulated CD4⁺CD45RA⁺T cells proportion and decreased CD4⁺CD45RO⁺T cells ratio ($P < 0.01$).

TCZ impact on Th17 cells proportion in peripheral blood

Th17 cells proportion was $1.53 \pm 0.46\%$ before treatment, and it declined significantly to $0.46 \pm 0.16\%$ after TCZ treatment ($P < 0.01$) (Figure 3).

TCZ impact on Treg cells ratio in peripheral blood

As shown Figure 4, TCZ treatment obviously increased Treg cells ratio in peripheral blood, from $1.84 \pm 0.63\%$ to $5.53 \pm 1.62\%$ ($P < 0.01$).

Discussion

RA is a chronic systemic inflammatory disease mainly involving bone, synovial joints, and ligaments. Severe RA patients may be affected in all organs [1–3]. In addition, it also can produce effects in cardiovascular, lung, and blood systems [17]. The incidence of RA in adults is about 1–2% [18]. Multiple types of innate immune and acquired immune cells are involved in the RA process [4]. CD4 T cell abnormality was confirmed to be the main cause of RA in human and mouse studies [5–7]. In addition, Th17 cells and Treg cells also play an important role in RA [9,10]. Because RA mainly involves the joints, it has high morbidity and seriously affects ability to work and perform activities of daily living. Furthermore, RA can shorten life span and imposes a huge burden on patients and society. Thus, there is an urgent need to find effective drug treatments for RA in clinical application.

Currently, commonly used biological agents for RA in clinical settings include tumor necrosis factor (TNF)- α inhibitor (etanercept and infliximab) and recombinant humanized IL-6 receptor monoclonal antibody TCZ (ACTEMRA) [19,20]. TNF- α

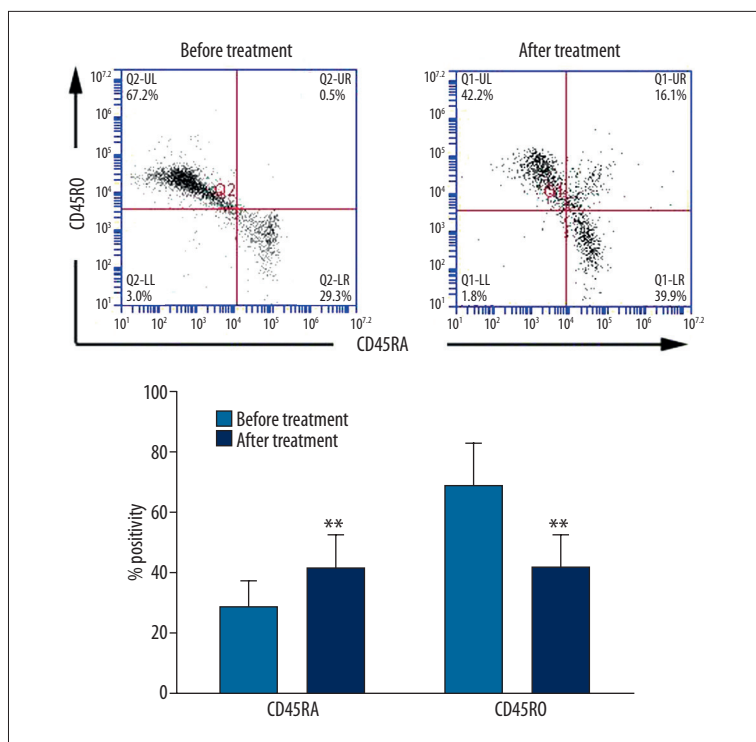


Figure 2. TCZ impact on CD4 T cells ratio in peripheral blood. ** $P < 0.01$ (** compared with before treatment).

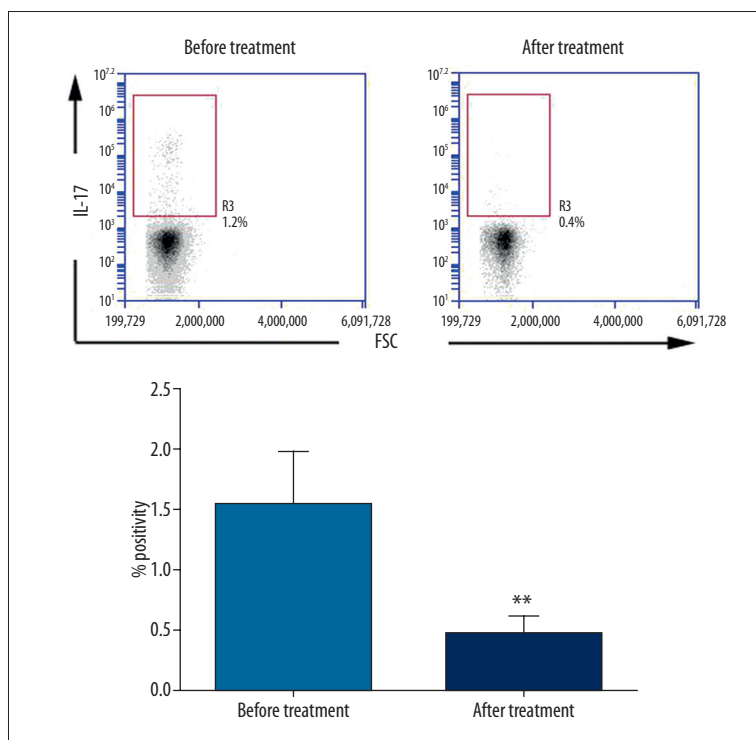


Figure 3. TCZ impact on Th17 cells proportion in peripheral blood. ** $P < 0.01$ (** compared with before treatment).

inhibitor was explored early-on and its mechanism is relatively clear. Research showed that Treg cells' immunosuppression function in RA patients' synovia was significantly reduced by Foxp3 phosphorylation [20]. TNF- α inhibitor treatment obviously changed Foxp3 phosphorylation and recovered Treg cells'

immunosuppression function [20]. Shen et al. [21] revealed that TNF- α inhibitor significantly reduced Th17 cells ratio in peripheral blood and serum IL-17 level. TCZ was recently developed, and the mechanism by which it works in treating RA is not fully understood.

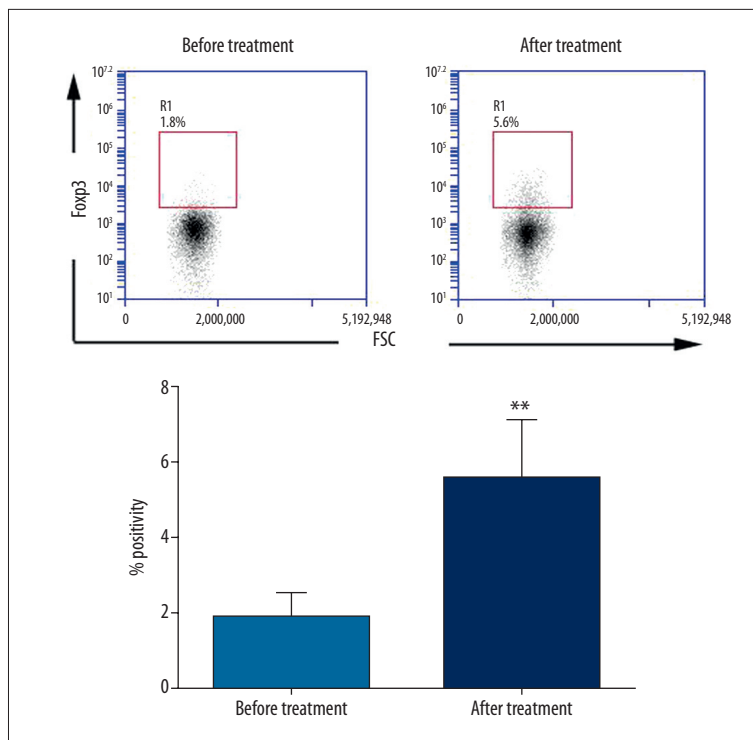


Figure 4. TCZ impact on Treg cells ratio in peripheral blood. ** $P < 0.01$ (** compared with before treatment).

CRP may increase during infection, injury, or inflammation, and its elevation in RA usually indicates active stage, while its decrease indicates stable stage. RF is the antibody of degenerated IgG induced by infection factors, and its elevation is closely associated with bone destruction. CCP shows good sensitivity and specificity to RA, especially in bone destruction. For CRP, RF, and CCP characteristics in RA, their levels can effectively reflect patient condition. This study showed that TCZ significantly improved patient condition by decreasing DAS28 score, CRP, RF, and CCP levels by more than half ($P < 0.01$). Our results are consistent with previous reports of TCZ remarkably improving RA patients at moderate and severe active stages [22].

We further analyzed the mechanism of TCZ in treating RA. It was reported that compared with healthy people, RA patients presented obviously lower CD4 naïve T cells proportion and elevated CD4 memory T cells ratio, suggesting that an imbalance between CD4 naïve T cells and CD4 memory T cells is closely associated with autoimmune disease [23]. Our results confirmed that, compared with before treatment, TCZ significantly increased CD4 naïve T cells percentage and decreased

CD4 memory T cells ratio ($P < 0.01$), indicating that TCZ can improve RA condition through regulating the balance between CD4 naïve T cells and CD4 memory T cells. In addition, an imbalance in the proportion Th17 cells and Tregs cells in peripheral blood was related to RA, presenting as Th17 cells ratio upregulation and Treg cells ratio reduction [9,24]. Our results revealed that TCZ obviously decreased Th17 cells proportion and Treg cells percentage in peripheral blood ($P < 0.01$).

Conclusions

TCZ can be used to treat RA patients through regulating the ratio of CD4 naïve T cells, CD4 memory T cells, Th17 cells, and Treg cells in peripheral blood.

Conflicts of interest

The authors declare they have no competing financial or commercial interests in this manuscript.

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