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Interleukin 6 receptor inhibitor tocilizumab suppresses cytokine expression, inflammasome activation and phagocytosis in a cell model of sepsis

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Objective: Sepsis is a life-threatening condition, usually accompanied by excessive inflammation. Tocilizumab (TCZ) is a humanized monoclonal antibody against the interleukin (IL) 6 receptor and has been studied in various inflammatory diseases, but little is known about its effects in sepsis. This study aims to reveal the role of TCZ in inflammation during sepsis. *Methods:* Human monocyte cell line THP-1 was stimulated by lipopolysaccharide (LPS) as a cell model for sepsis. After TCZ treatment, the expression of cytokines tumor necrosis factor (TNF) and IL10, the production of chemokine (C-C motif) ligand 2 (CCL2) and IL1B, and the expression of inflammasome factors NLR family pyrin domain containing 3 (NLRP3) and caspase 1 (CASP1), were detected by qRT-PCR and ELISA. Phagocytosis assay was also performed to assess the phagocytosis activity of TCZ-treated cells. *Results:* LPS stimulation significantly upregulated *TNF* and *IL10* mRNA levels ($P < 0.01$) and CCL2 and IL1B production ($P < 0.001$), promoted *NLRP3* and *CASP1* levels ($P < 0.01$) and elevated phagocytosis activity of THP-1 cells ($P < 0.001$). TCZ treatment had the opposite effects of decreasing *TNF* and *IL10* mRNA levels ($P < 0.05$), CCL2 and IL1B production ($P < 0.001$), inhibiting *NLRP3* and *CASP1* ($P < 0.01$), and suppressing phagocytosis activity ($P < 0.001$) compared to the LPS group. *Conclusion:* These results indicate the suppressive role of TCZ in cytokine production, inflammation activation and phagocytosis in sepsis cell model, implying its effects on controlling "cytokine storm" during sepsis. Thus TCZ provides a promising strategy for treating sepsis.

1. Introduction

Sepsis is a life-threatening condition caused by infection-triggered immune response. Severe sepsis may lead to multi-organ dysfunction syndrome or multiple organ failure, constituting a major reason for the high death rate. A study has detected an estimated incidence of sepsis of about 4.4 % in a general population of patients admitted to hospital (Esteban et al. 2007). However, the incidence of sepsis is still increasing in the past decade, with the increasing rate ranging from 54% to 71% (Rhee et al. 2015), which calls for immediate and effective therapy to improve treatment outcomes.

Sepsis is usually accompanied by excessive inflammation, which sometimes develops into a "cytokine storm", and then followed the decreased functioning of the immune system (Harrison 2010). Cytokine storm is used to describe the abrupt release of various cytokines by the immune system in response to infection, but it may cause disruptions in the physiology of patients (London et al. 2010). Inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukins (ILs), are crucial for the stabilization of endothelial cell interactions, and thus their elevated production are dangerous in sepsis (Mándi et al. 1995; Aird 2003; Barkhausen et al. 2009). From this point of view, controlling the levels of soaring cytokines is promising for relieving the syndromes of sepsis.

IL6 is a kind of interleukin that has been revealed correlated with the severity of several diseases (Tyburski et al. 2001). Its elevation in the plasma of sepsis patients has been considered helpful to indicate the prognosis of sepsis treatment (Patel et al. 1994) and predict postoperative sepsis in patient who have received major surgery for cancer (Pettilä et al. 2002; Mokart et al. 2005). Further, repression of the IL6 level shows pivotal therapeutic implications in a sepsis cell model (Galley et al. 1997), which may be a possible strategy to control the severity of inflammation in sepsis. Tocilizumab (TCZ) is a humanized monoclonal antibody against IL6 receptor (IL6R), blocking IL6 signaling via inhibiting the interaction of IL6 and IL6R (Tanaka et al. 2011). It has shown great effects in various autoimmune and inflammatory diseases, such as rheumatoid arthritis (Inoue et al. 2010), systemic sclerosis (Shima et al. 2010) and polymyositis (Okiyama et al. 2009). However, little is known about its effect on sepsis.

This study focused on the role of TCZ in inflammation during sepsis. Because of the involvement of monocytes in activation of inflammatory factors during sepsis (Barlage et al. 2009), human monocyte cell line THP-1 was stimulated by lipopolysaccharide (LPS) as a cell model for sepsis. Cytokine levels, including the mRNA levels of *TNF* and *IL10*, the concentration of chemokine (C-C motif) ligand 2 (CCL2) and IL1B in the culture medium, and mRNA expression of inflamasome factors NLR family pyrin domain containing 3 (*NLRP3*) and caspase 1 (*CASP1*), were detected. Phagocytosis activity of the treated cells was assessed. These results will help to reflect the effect of TCZ on cytokine production, inflammation activation and phagocytosis activity in sepsis, and facilitate the research of new strategies on treating sepsis.

2. Investigations and results

2.1. TCZ reduces cytokine expression and release from LPS-induced THP-1 cells

First, the effect of TCZ on cytokines in the sepsis cell model was analyzed. The mRNA levels of cytokine *TNF* and *IL10* in THP-1 cells were both significantly upregulated compared to the control group ($P < 0.01$, Fig. 1A and 1B). TCZ had obvious influences

on these cytokines, significantly impairing their mRNA levels compared to the LPS group ($P < 0.05$). Thus TCZ could affect *TNF* and *IL10* in the LPS-induced cell model of sepsis.
Besides, the concentration of cytokine CCL2 and IL1B in the culture medium was detected by ELISA, and results indicated the markedly increase of both cytokines in the LPS groups compared to the control group ($P < 0.001$, Fig. 2A and 2B), as well as the significantly suppressed levels by TCZ treatment ($P < 0.001$ for CCL2 and $P < 0.01$ for IL1B). Thus TCZ could also control CCL2 and IL1B concentration in the culture medium, which might represent that TCZ inhibited CCL2 and IL1B production from the THP-1 cells.

2.2. *TCZ suppresses inflammasome activation and phagocytosis of LPS-induced THP-1 cells*

Next, we assessed the degree of inflammasome activation, as indicated by the mRNA levels of *NLRP3* and *CASP1*. qRT-PCR showed the two factors were both upregulated in the LPS groups ($P < 0.01$ for *NLRP3* and $P < 0.001$ for *CASP1*, Fig. 3A and 3B) and suppressed by TCZ treatment ($P < 0.01$), which implied that TCZ might affect inflammasome activation of the LPS-induced THP-1 cells.
We also examined phagocytosis activity of monocytes after LPS stimulation. The percent of cells ingesting FITC-labeled dextran was increased by LPS ($P < 0.001$, Fig. 4), and decreased by TCZ ($P < 0.001$), indicating that TCZ could inhibit the phagocytosis activity of monocytes in the cell model of sepsis.

3. Discussion

Severe sepsis is usually accompanied by cytokine storm, which may affect functioning of multiple organs of patients. In this study, we found that TCZ, a humanized monoclonal antibody against IL6R, was capable of controlling *TNF* and *IL10* mRNA levels, CCL2 and IL1B production, *NLRP3* and *CASP1* levels and phagocytosis in the LPS-induced THP-1 cell model of sepsis.
The mRNA levels of *TNF* and *IL10* in the cell model were detected via qRT-PCR. TNF is a crucial inflammatory cytokine and its expression is triggered in LPS-induced THP-1 cells (Kim, 2010), as well as in sepsis patients, which may result from the higher polymorphism of *TNF* gene (Kothari et al. 2013). Studies have found TNF as a useful early diagnostic marker for sepsis (Ucar et al., 2008) and that inhibiting TNF has marked effects of reducing the risk of dying in sepsis patients (Qiu et al., 2013). As for IL10, a cytokine with complex roles in sepsis, its production by LPS-induced THP-1 cells was promoted by TNF (Khoa et al. 2001). Our results showed the phenomenon consistent with former studies

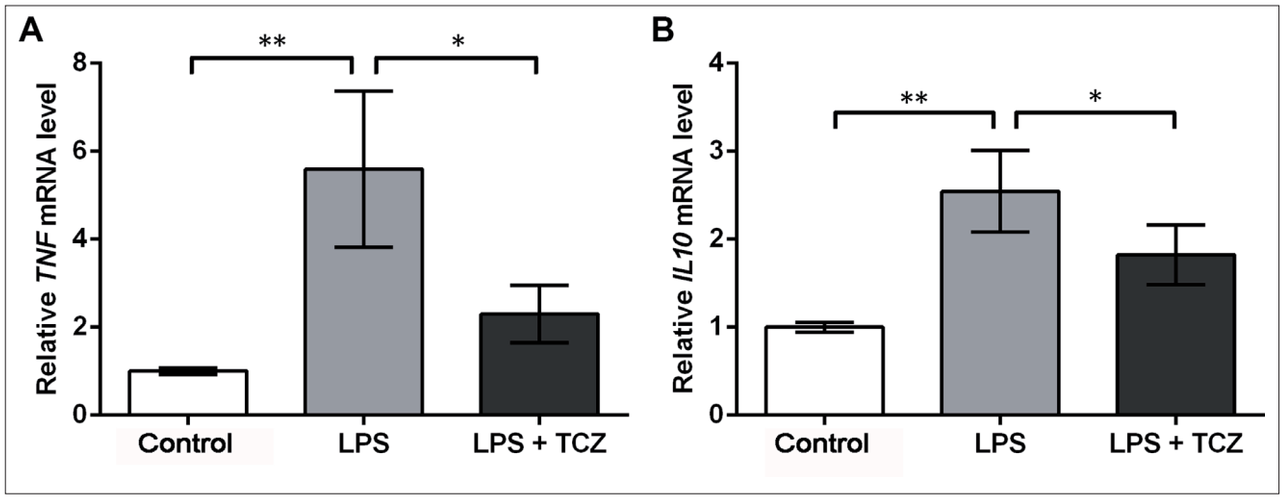

Fig. 1: The lipopolysaccharide (LPS)-induced mRNA levels of tumor necrosis factor (*TNF*) and interleukin 10 (*IL10*) in THP-1 cells are suppressed by tocilizumab (TCZ). Human monocyte THP-1 cells were treated with LPS (10 μg/mL) or LPS plus TCZ (10 μg/mL) for 24 h, after which the mRNA levels of *TNF* and *IL10* were detected by qRT-PCR. * $P < 0.05$. ** $P < 0.01$.

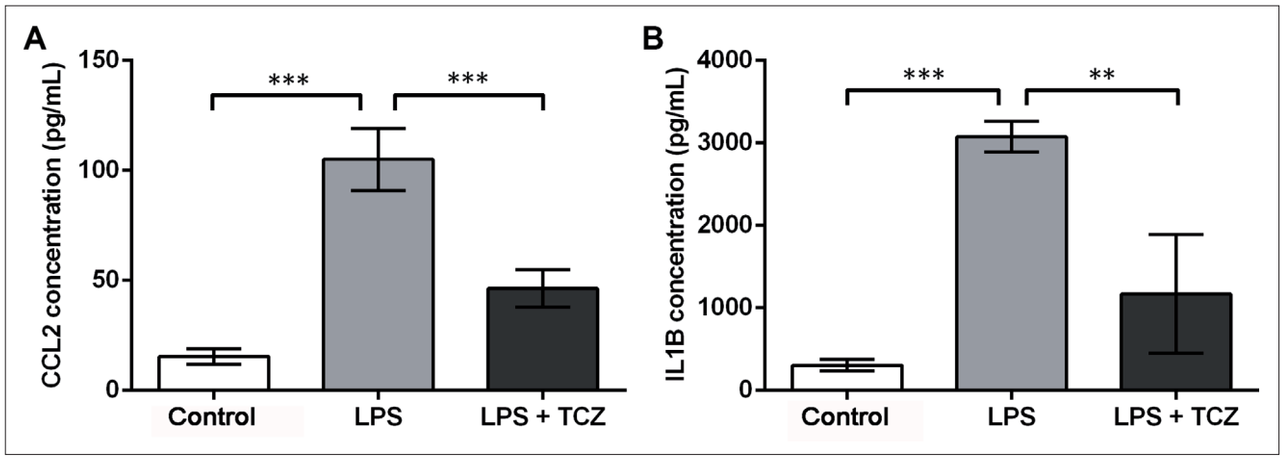

Fig. 2: The lipopolysaccharide (LPS)-induced chemokine (C-C motif) ligand 2 (CCL2) and interleukin 1β (IL1B) levels in the culture medium of THP-1 cells are suppressed by tocilizumab (TCZ). Human monocyte THP-1 cells were treated with LPS (10 μg/mL) or LPS plus TCZ (10 μg/mL) for 24 h, after which the levels of CCL2 and IL1B in the culture medium were detected by ELISA. ** $P < 0.01$. *** $P < 0.001$.

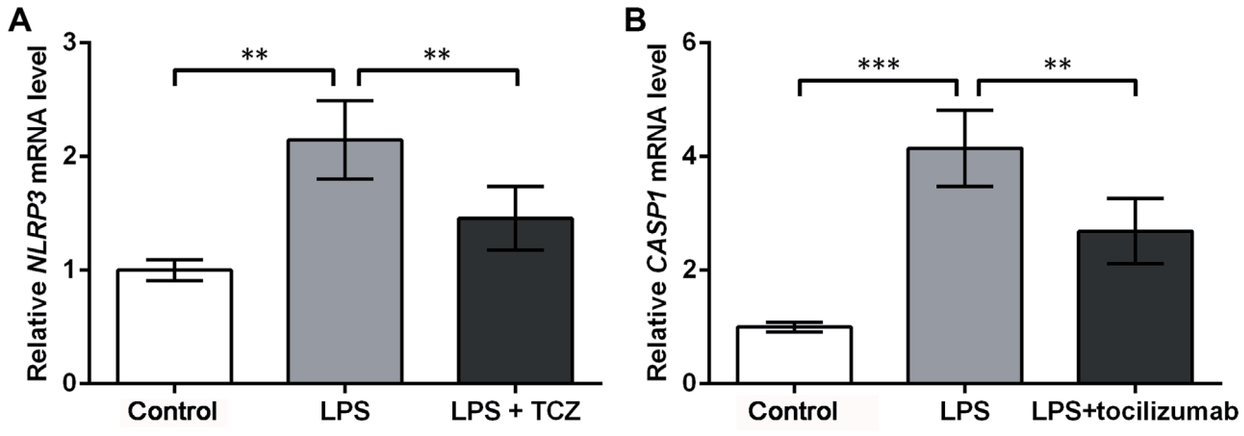

Fig. 3: The lipopolysaccharide (LPS)-induced mRNA levels of inflammasome factors NLR family, pyrin domain containing 3 (*NLRP3*) and caspase 1 (*CASP1*) in THP-1 cells are suppressed by tocilizumab (TCZ). Human monocyte THP-1 cells were treated with LPS (10 μg/mL) or LPS plus TCZ (10 μg/mL) for 24 h, after which the mRNA levels of *NLRP3* and *CASP1* were detected by qRT-PCR. ** $P < 0.01$. *** $P < 0.001$.

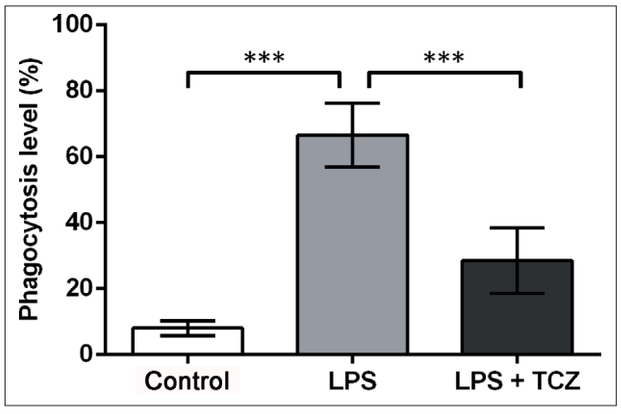

Fig. 4: The lipopolysaccharide (LPS)-induced phagocytosis of THP-1 cells is suppressed by tocilizumab (TCZ). Human monocyte THP-1 cells were treated with LPS (10 μg/mL) or LPS plus TCZ (10 μg/mL) for 24 h, after which the cells were co-incubated with FITC-Dextran for 6 h. Cells were detected by flow cytometry and the percent of FITC-positive cells were calculated to indicate the phagocytosis level. *** $P < 0.001$.

that the mRNA levels of *TNF* and *IL10* were promoted in the LPS-induced THP-1 cells. We further found the two mRNAs were suppressed by TCZ treatment, suggesting that TCZ may inhibit the expression of these cytokines in monocytes during sepsis.

Similarly, the production of CCL2 and IL1B were examined by ELISA as representatives for major cytokines, since the two factors are primarily secreted from monocytes. CCL2 is also mentioned as monocyte chemoattractant protein 1, recruiting monocytes and macrophages to the site of inflammation (Schober et al. 2004; Shen et al. 2014). Both CCL2 and IL1B are pro-inflammatory factors, whose release from THP-1 cells is induced by LPS treatment (Parmentier et al. 2000; Gusman et al. 2015). We found the increased concentration of CCL2 and IL1B in the culture medium of the LPS-induced THP-1 cells, which is in line with former studies, while TCZ treatment controlled the LPS-induced CCL2 and IL1B, suggesting that TCZ may inhibit the production of the two cytokines from monocytes during sepsis. Together with the abovementioned *TNF* and *IL10* regulation by TCZ, it is implied that TCZ is capable of suppressing cytokines in the THP-1 cell model of sepsis.

In addition to *TNF* and *IL10*, the qRT-PCR experiments also detected the elevated mRNA levels of *NLRP3* and *CASP1* in the LPS-induced THP-1 cells, which were decreased by TCZ treatment. NLRP3 and CASP1 are two major components of the NLRP3 inflammasome: NLRP3 encodes the sensor component of the inflammasome and is an activator of CASP1 (Paugh et al. 2015). Besides, NLRP3 is a possible target for sepsis treatment since its knock-down in the rat model alleviates sepsis-induced hyper-bileacidaemia (Wu et al. 2015). Similarly, CASP1 inhibitor has also been indicated promising in protecting against sepsis (Matute-Bello 2007). Thus the suppressed expression of *NLRP3* and *CASP1* represents the inhibition of inflammasome activation, and the inhibition of the two factors by TCZ may imply the capability of TCZ in blocking inflammasome activation during sepsis.

Inflammatory monocytes can be transferred to the sites of inflammation and produce inflammatory cytokines. Moreover, they are capable of differentiating into macrophages, which participate in systemic inflammation (Yang et al. 2014). Here the phagocytosis activity of the sepsis cell model was examined, and results showed that LPS induced phagocytosis, possibly due to the induced differentiation from monocytes to macrophages, which is similar to existed studies in THP-1 cells (Baqui et al. 1998; Michée et al. 2013), while TCZ significantly suppressed phagocytosis of LPS-induced THP-1 cells, implying its roles of controlling phagocytosis of monocytes during sepsis.

TCZ has been tested in patients of various diseases and relatively satisfactory outcomes are acquired in arthritis, for example, where TCZ is effective as a combination therapy (Mori et al. 2013; Horneff et al. 2015). However, little is known about the possible usage of TCZ in sepsis. This *in vitro* study implicates that TCZ has the potential of inhibiting cytokine expression and production, inflammasome activation and phagocytosis in the LPS-induced cell model of sepsis, which may imply its potential usage as therapeutic alternatives or adjuvant therapy to suppress the "cytokine storm" in sepsis. Further *in vivo* studies are necessary to fully understand the effect of TCZ in sepsis.

4. Experimental

4.1. Cells and treatment

Human monocytes THP-1 (ATCC, Manassas, VA) were cultured in Roswell Park 1640 (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Shanghai, China). Cells were incubated in humidified atmosphere containing 5% CO_2 at 37 °C. The culture medium was changed every three days. LPS was used to induce sepsis cell model in THP-1 cells. The cells were divided into three groups and plated in 6-well plates (1×10^6 cells per well), namely, control, LPS, and LPS + TCZ. Cells in the LPS group were treated by 10 μg/mL LPS (Sigma-Aldrich) for 24 h (Musenster et al. 2015), and cells in the LPS + TCZ group were co-stimulated by TCZ (10 μg/mL) for 24 h (Betts et al. 2011). Culture medium was replaced after treatment and the cells were collected for further analyses.

4.2. Phagocytosis assay

The phagocytosis activity of THP-1 cells was measured after the 24-h treatment by LPS and TCZ. Briefly, the cells were collected, washed in culture medium and co-incubated with fluorescein isothiocyanate (FITC)-labeled Dextran (10,000 kDa, Nanocs, Boston, MA) for 6 h according to the manufacturer's instructions. Then the cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 10 min. Flow cytometry was conducted on BD FACSCanto II (BD Biosciences, San Jose, CA) to analyze the cells, and the phagocytosis level was calculated as (number of FITC-positive cells)/(number of total cells).

4.3. ELISA

ELISA was performed to detect the concentration of CCL2 and IL1B in the culture medium of THP-1 cells using commercial kits (Abnova, Beijing, China) according to the manufacturer's instructions. Briefly, 100 μL of the culture medium was added to each well of the plate, which was then incubated at room temperature for 1 h. Then biotinylated antibodies and streptavidin-horse radish peroxidase (HRP) was added successively, with 3 times of rinse by washing buffer. Signals were developed in tetramethylbenzidine (TMB) with incubation in the dark for 15 min. After the reaction was terminated, the signals were detected under a measurement wavelength of 450 nm using microplate reader Multiskan Go (Thermo Scientific, Carlsbad, CA).

4.4. qRT-PCR

The cells of each group were collected and immediately used for total RNA extraction with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNA contamination was removed by DNase I (Invitrogen), and the quality and quantity of RNA samples were examined by Nanodrop 2000 (Thermo Scientific). For each sample, 1 μg of total RNA was used in the reverse-transcription reaction catalyzed by SuperScript III Reverse Transcriptase (Invitrogen). qRT-PCR was performed on QuantStudio 6 Flex Realtime PCR system (Applied Biosystem, Carlsbad, CA) with 20 ng of complementary DNA (cDNA) and the specific primers for *TNF* (Fw: 5'-GGCCA AGCCC TGGTA TGAG-3' and Rv: 5'-TAGTC GGGCC GATTG ATCTC-3'), *IL10* (Fw: 5'-AAAAG AAGGC ATGCA CAGCT CAG-3' and Rv: 5'-GTGGG TGCAG CTGTT CTCAG ACT-3'), *NLRP3* (Fw: 5'-GAAGA AAGAT TACCG TAAGA AGTAC AGAAA-3' and Rv: 5'-CGTTT GTTGA GGCTC ACACT CT-3') and *CASP1* (Fw: 5'-TGTTC CTGTG ATGTG GAGGA-3' and Rv: 5'-TCTTT CAGTG GTGGG CATCT-3'). *GAPDH* (Fw: 5'-GAAGG TGAAG GTCGG AGTC-3' and Rv: 5'-GAAGA TGGTG ATGGG ATTTG-3') was used as an internal reference gene. The result of each sample was normalized by *GAPDH* and then compared to the control group. Data were calculated by $2^{-\Delta\Delta Ct}$ method.

4.5. Statistical analysis

All the experiments were repeated five times and the results were represented as mean±standard deviation. Data were analyzed by *F* test for homogeneity of variance followed by *t* test in SPSS 20 (IBM, New York, NY). Date with significant difference between groups were identified when $P < 0.05$.

Conflicts of interest: None declared.

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