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Original Article

TLR4-dependent internalization of CX3CR1 aggravates sepsis-induced immunoparalysis

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Abstract: Sepsis, the most severe manifestation of infection, poses a major challenge to health-care systems around the world. Limited ability to clean and remove the pathogen renders difficulty in septic patients to recover from the phase of immunoparalysis. The present study found the vital role of CX3CR1 internalization on sepsis-induced immunoparalysis. A mouse model with cecal ligation and puncture (CLP) and cell model with lipopolysaccharides (LPS) were employed to explore the relationship between CX3CR1 internalization and septic immunoparalysis. Immunoparalysis model in mice was established 4 days after CLP with significantly decreased proinflammatory cytokines. Flow cytometry analysis found a decreased surface expression of CX3CR1 during immunoparalysis, which was associated with reduced mRNA level and increased internalization of CX3CR1. G-protein coupled receptor kinase 2 (GRK2) and β-arrestin2 were significantly increased during septic immunoparalysis and involved in the internalization of CX3CR1. TLR4$^{-/-}$ or TLR4 inhibitor-treated macrophages exhibited an inhibited expression of GRK2 and β-arrestin2, along with reduced internalization of CX3CR1. Moreover, the knockdown of GRK2 and β-arrestin2 inhibited the internalization of CX3CR1 and led to a higher response on the second hit, which was associated with an increased activation of NF-κB. The critical association between internalization of CX3CR1 and immunosuppression in sepsis may provide a novel reference for clinical therapeutics.

Keywords: Sepsis, immunoparalysis, internalization, CX3CR1, TLR4

Introduction

Sepsis is a severe systemic bacterial infection, which is defined as widespread inflammation secondary to infections; the incidence of sepsis in critically ill patients has been continuously increasing over the last two decades [1]. Due to complex host immune, inflammatory, and coagulation responses to infecting microbes, sepsis frequently results in lethal shock with coagulopathy and multiple organ failure. Despite major advances in the supportive therapy, it continues to remain as the leading cause of admission and death in intensive care units [2]. In the acute phase, the cytokine storm (an exaggerated systemic release of cytokines) leads to hypotension, cardiovascular dysfunction, tissue damage, and multi-organ failure [3]. However, during the later phases of the disease, the activation of modulatory pathways can prematurely inhibit the host defense, which is universally known as innate immunotolerance/immunoparalysis [4]. With current clinical therapy, a significant number of patients could survive from the "cytokine storm" to immunoparalysis. Owing to a limited ability to clear and remove the pathogen, patients in the immunoparalysis stage are susceptible to death [4, 5].

The pathophysiology of sepsis involves various components of innate immunity, especially mononuclear phagocytes. Monocytes/macrophages are speculated to generate multiple cytokines that trigger a chain reaction leading

to tissue damage and death. However, the mechanisms of immunoparalysis are not yet clear. Recently, studies on the mechanisms of immunoparalysis focused on apoptosis and dysfunction of immune cells, as well as, exaggerated the release of anti-inflammatory cytokines (for example, IL-4, IL-10, IL-13) and activation of regulatory T.cells [6]. A panel of genes was identified, among which, CX3C chemokine receptor 1 (CX3CR1) was found to be significantly upregulated in survivors as compared to non-survivors [7].

CX3CR1 is a G-protein coupled receptor that is expressed on T lymphocytes, mast cells, natural killer cells, dendritic cells, platelets, neurons, astrocytes, microglial cells, and monocytes [8]. Fractalkine is the sole member of the CX3C chemokine subfamily. The major roles of fractalkine/CX3CR1 lie in chemotactic activity, adhesion, and transmigration ability [9, 10]. CX3CR1-mediated inflammation has been demonstrated in several inflammatory disorders such as atherosclerosis and arthritis [11, 12]. The decreased CX3CR1 expression was reported to be an independent molecular biomarker of early and late mortality in critically ill patients [13]. However, various inflammatory injuries of lung and kidney during sepsis were associated with CX3CR1 [14, 15]. Moreover, CX3CR1 expression was severely downregulated in monocytes of septic shock patients [16]. Nevertheless, whether CX3CR1 influences the immunosuppression state during sepsis, is yet unknown. Herein, we found increased internalization of CX3CR1 in the late phase of sepsis, and further investigated the role of CX3CR1 internalization in septic immunoparalysis.

Materials and methods

Mouse strains

All the C57BL/6 (purchased from Shanghai SLAC Laboratory Animal CO. LTD, Shanghai, China) and TLR4 knock-out ($TLR4^{-/-}$) mice (purchased from Model Animal Research Center of Nanjing University, Jiangsu Province, China) used in the current experiments were 8-10-weeks-old, male, and bred in a temperature-controlled (22 ± 2°C) environment under a 12: 12 h light: dark cycle in the Animal Center of Secondary Military Medical University. All the animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of the Second Military Medical University (SMMU, Shanghai, China).

Cell isolation and culture

Peritoneal macrophages (PMs) were isolated by peritoneal lavage after injecting 2 mL sterile enriched thioglycolate broth for 3 days. The lavage fluid was centrifuged at 300 g for 10 min, and the pelleted cells were resuspended and cultured in DMEM containing 10% FBS supplemented with 50 μg/mL penicillin/streptomycin at 37°C in a humidified atmosphere (5% CO_2, 95% air). After incubation for 2 h, the non-adherent cells were washed off with PBS, followed by medium replacement. RAW264.7 cells were cultured in 1640 medium with 10% FBS and 50 μg/mL penicillin/streptomycin at 37°C in a humidified atmosphere (5% CO_2, 95% air).

LPS-induced cell model

After being isolated and cultured in 12-well dishes overnight, PMs (1×10^6/well) were treated with LPS (100 ng/mL, Sigma) or PBS for 3 h and 24 h, respectively. Then, the cells were collected and prepared for analysis. For the "two-hit" model, PMs or RAW264.7 cells were treated with LPS (100 ng/mL, Sigma) for 12 h. After refreshing the medium and culturing for 24 h, the cells were treated with LPS (100 ng/mL) for 3 h ("second hit") and then harvested for analysis.

CLP surgery

Mice were anesthetized with an i.p. injection of pentobarbital (50 mg/kg). A 1.2-cm-long incision was made to the lower left abdomen, cecum exposed, ligated tightly (1/3 of cecum) with silk sutures below the ileocecal valve, and punctured through twice with an 18-gauge needle. Then, the cecum was returned into the peritoneal cavity, and the incision was closed. Subsequently, the mice were subcutaneously injected with 1 mL sterile PBS to avoid dehydration and warmed on a heating pad until they recovered from anesthesia. For sham-treated animals, a similar procedure was performed, but without ligation and puncture.

RNA isolation and quantitative real-time PCR

Total RNA from cells or tissue was extracted using TRIzol reagent (Invitrogen). RNA was converted to cDNA with Superscript II reverse transcriptase following the manufacturer's instructions. Each sample was tested in triplicate for analysis of relative gene expression, and the

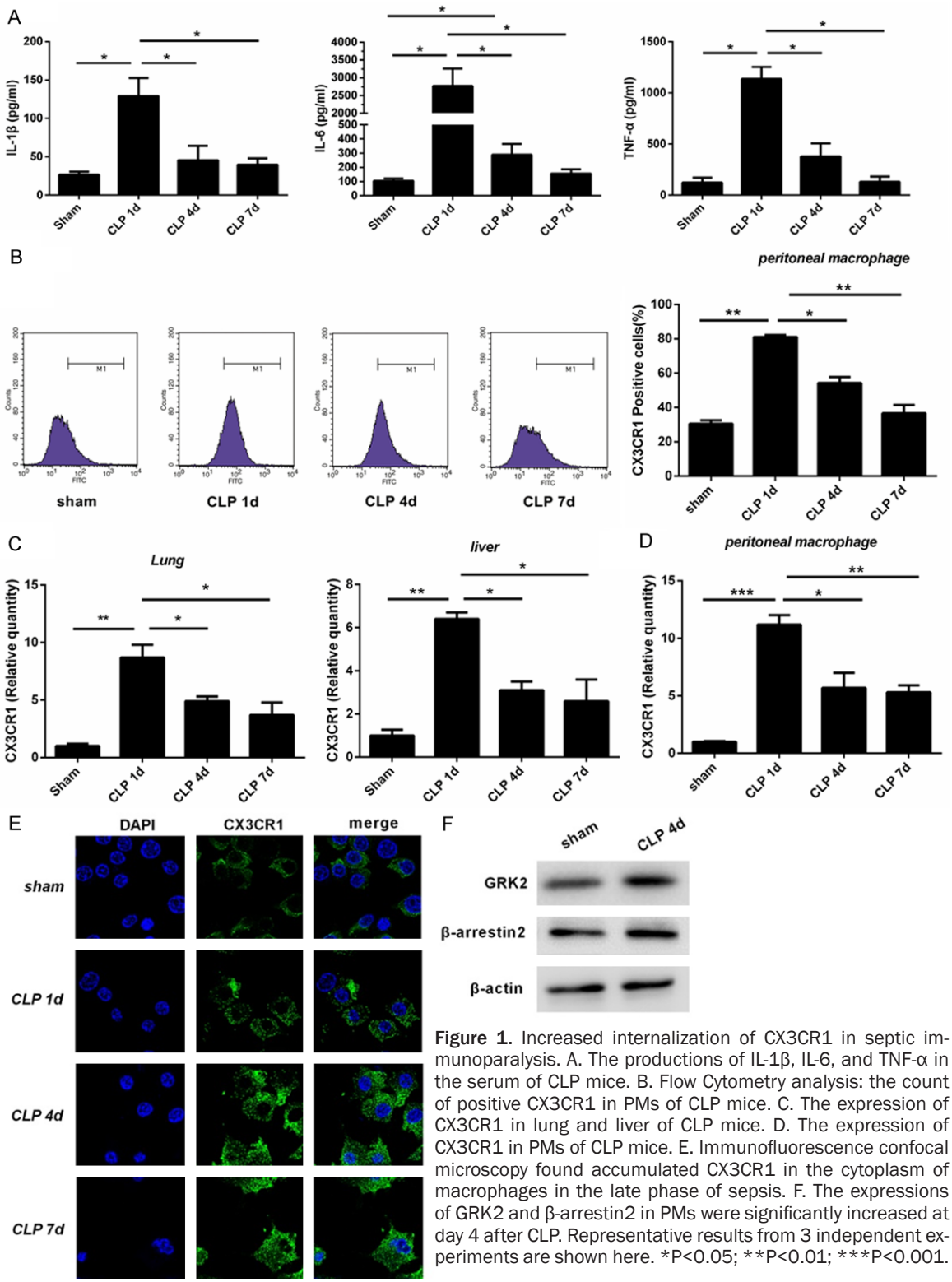

Figure 1. Increased internalization of CX3CR1 in septic immunoparalysis. A. The productions of IL-1β, IL-6, and TNF-α in the serum of CLP mice. B. Flow Cytometry analysis: the count of positive CX3CR1 in PMs of CLP mice. C. The expression of CX3CR1 in lung and liver of CLP mice. D. The expression of CX3CR1 in PMs of CLP mice. E. Immunofluorescence confocal microscopy found accumulated CX3CR1 in the cytoplasm of macrophages in the late phase of sepsis. F. The expressions of GRK2 and β-arrestin2 in PMs were significantly increased at day 4 after CLP. Representative results from 3 independent experiments are shown here. *P<0.05; **P<0.01; ***P<0.001.

transcript levels of β-actin were monitored as an internal control. Primers used in this study were as follow: β-actin, forward: 5'-AGTGTGACGTTGACATCCGT-3', reverse: 5'-GCAGCTCAGTAACAGTCCGC-3'; CX3CR1, forward: 5'-CTTCTGGTGGTCACGTGTT-3', reverse: 5'-GGTATCTTCTGAACTTCTCCC-3'; β-arrestin2, forward: 5'-CGATACCCCAAAATTTGCCTTG-3', reverse: 5'-TG-

ATAAGCCGCACAGAGTTC-3'; GRK2, forward: 5'-CTGCCAGAGCCCAGCATC-3', reverse: 5'-AGAAGTCCCGGAAAAGCAGG-3'.

Western blot

Tissue extracts were separated by 10% SDS-PAGE followed by transferring to PVDF membranes. After blocking for 1 h at room temperature, the blots were incubated with rabbit polyclonal antibodies against GRK2, β-arrestin2, p65/p-p65, IκB/p-IκB, and β-actin at 4°C overnight. The membranes were then washed with TBST three times and incubated with corresponding secondary antibodies. The immunoreactive bands were detected using Odyssey System.

siRNA transfection

For siRNA treatment in RAW264.7, oligonucleotides corresponding to nucleotide sequences of CX3CR1, GRK2, and β-arrestin2 were synthesized commercially by Invitrogen and used at the concentration of 100 nM. A scrambled oligonucleotide was utilized as the negative control concurrently.

Flow cytometry analysis

PMs treated with LPS at indicated time-points were suspended in ice-cold PBS. Cells were washed twice with fluorescence-activated cell sorting (FACS) buffer (PBS/2% fetal calf serum) and resuspended in 100 μL of ice-cold FACS buffer. Cells were incubated with FITC-conjugated polyclonal antibody against CX3CR1 at 4°C 30 min following the manufacturer's instructions, followed by flow cytometry. SSC and FSC were used to differentiate the cells. Background and auto-fluorescence were determined by a control antibody with the same isotype staining. Acquisition was performed on 10000 events using an FACSCalibur (R&D Systems) and analyzed by FlowJo-V10 software (Tree Star, Ashland, OR, USA).

Enzyme linked immunosorbent assay (ELISA)

At indicated time points, the culture supernatants and serum of CLP mice were obtained to determine the levels of a cytokine using commercial ELISA kits (R&D Systems). The detection limit of each method was as follows: IL-1β >3 pg/mL, IL-6 >2 pg/mL, TNF-α >9 pg/mL.

Immunofluorescence confocal microscopy

Cells were plated onto microscopic slides and fixed with 4% paraformaldehyde for 10 min. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, followed by blocking with 3% bovine serum albumin in PBST (PBS with 0.2% Tween-20) for 2 h at room temperature to reduce non-specific staining. Subsequently, the cells were incubated with the primary rabbit polyclonal antibody against CX3CR1 at 4°C overnight. After washing twice with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. After washing with PBS for three times, the cells were analyzed by confocal microscopy.

Data presentation and statistical analysis

The data are presented as mean ± standard deviation (SD). SPSS18.0 and GraphPad Prism were used for statistical analysis and figures representation. Significances between groups were determined by one-way ANOVA or two-tailed Student's t-test. The *P-value* <0.05 was considered statistically significant.

Results

Increased internalization of CX3CR1 in septic immunoparalysis

The mice entered the stage of immunoparalysis 4 days after CLP with significantly decreased inflammatory cytokines, including IL-1β, IL-6, and TNF-α (**Figure 1A**). Flow cytometry analysis found a significant reduction of CX3CR1 on the membranes during this period in CLP mice (**Figure 1B**), along with decreased expression of CX3CR1 (**Figure 1C** and **1D**). Immunofluorescence confocal microscopy was later employed to visualize the internalization as a critical mechanism for receptors diminution and found accumulated CX3CR1 in the cytoplasm of macrophages 4 days after CLP surgery (**Figure 1E**). Additionally, endocytosis-related proteins, GRK2 and β-arrestin2, were significantly increased during septic immunoparalysis (**Figure 1F**).

GRK2 and β-arrestin2 were involved in the internalization of CX3CR1

To further confirm the internalization phenomenon during immunoparalysis, LPS-induced

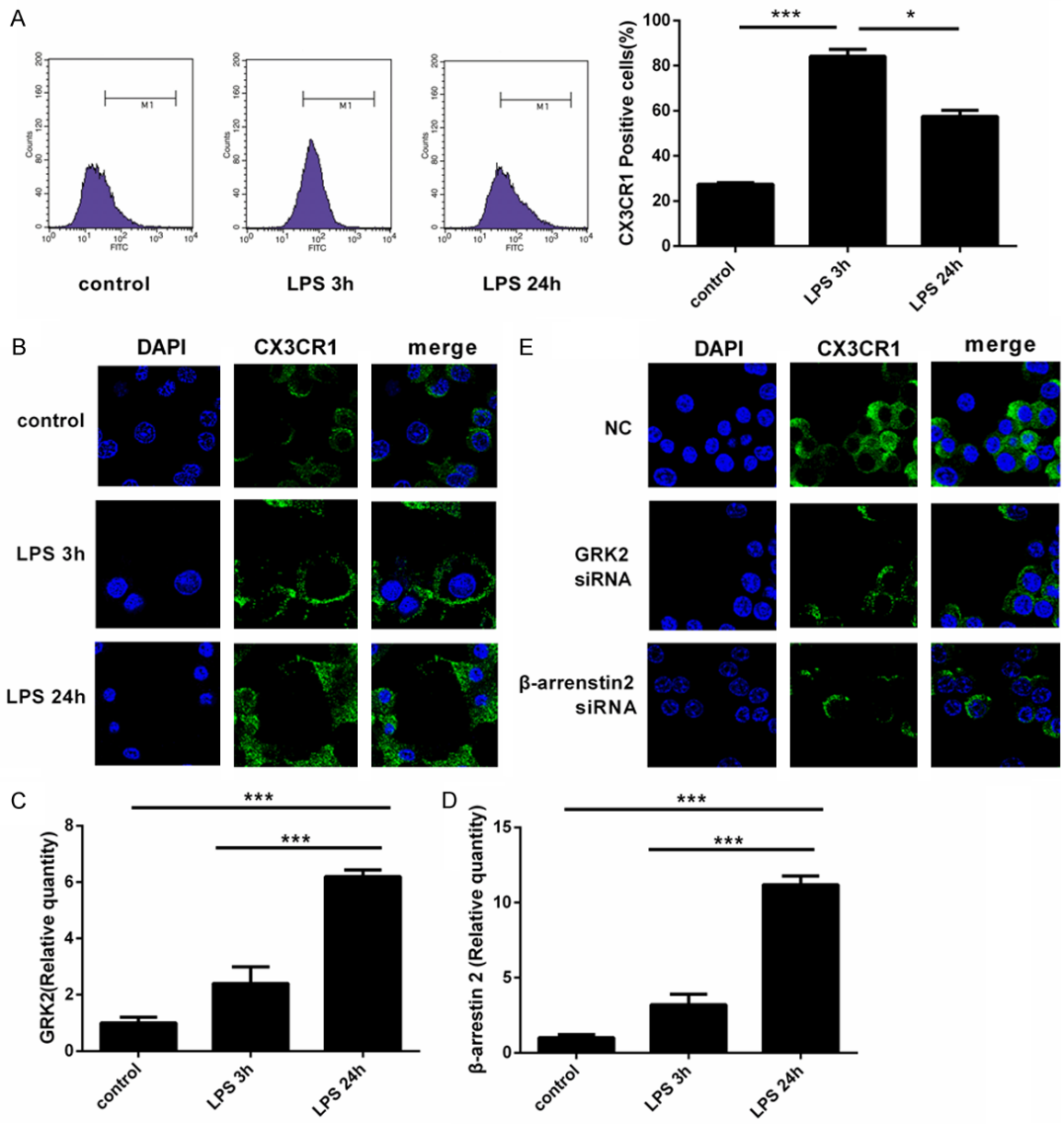

Figure 2. GRK2 and β-arrestin2 were involved in the internalization of CX3CR1. A. Flow Cytometry analysis: the count of CX3CR1 positive cells in LPS-treated PMs. B. Immunofluorescence confocal microscopy found accumulated CX3CR1 in the cytoplasm of macrophages stimulated with LPS for 24 h. C. The expression of GRK2 in LPS-treated PMs. D. The expression of β-arrestin2 in LPS-treated PMs. E. Knockdown of GRK2 and β-arrestin2 resulted in decreased internalization of CX3CR1. Representative results from 3 independent experiments are shown in here. Values represent means ± SD (n=3). NC, negative control; *P<0.05; **P<0.01; ***P<0.001.

endotoxin tolerance in the cell model was employed. A continually decreased expression of CX3CR1 was found on the membranes and increased internalization (**Figure 2A**, **2B**). Similarly, LPS treatment for 24 h resulted in augmented expression of both GRK2 and β-arrestin2 (**Figure 2C**, **2D**). Moreover, GRK2 and β-arrestin2 RNAi reduced the internalization of CX3CR1 (**Figure 2E**).

Internalization of CX3CR1 was dependent on intact TLR4

TLR4 is known as one of the most important receptors involved in sepsis, and is found to influence the expression of GRK2. Thus, we further investigated whether TLR4 contributed to the internalization of CX3CR1 in the late phase. TLR4-deficienct macrophages stimulated with

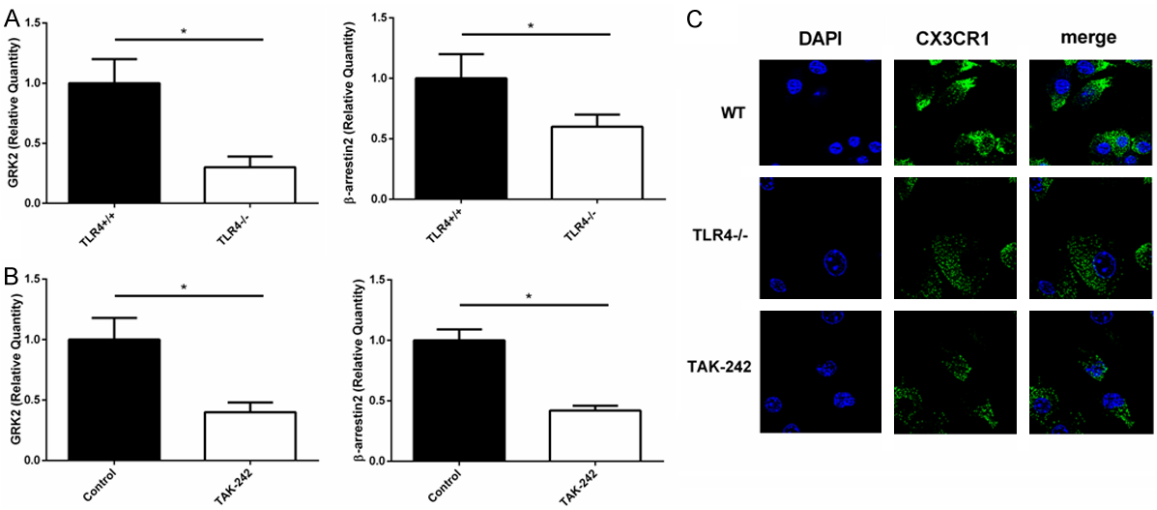

Figure 3. TLR4 deficiency led to reduced internalization of CX3CR1. A. TLR4$^{-/-}$ macrophages stimulated with LPS for 24 h resulted in decreased expression of GRK2 and β-arrestin2. B. TLR4 inhibitor (TAK-242) reduced the expression of GRK2 and β-arrestin2 in treated macrophages. C. Internalization of CX3CR1 in TLR4$^{-/-}$ and TAK-242-treated macrophages after LPS stimulated for 24 h. *P<0.05.

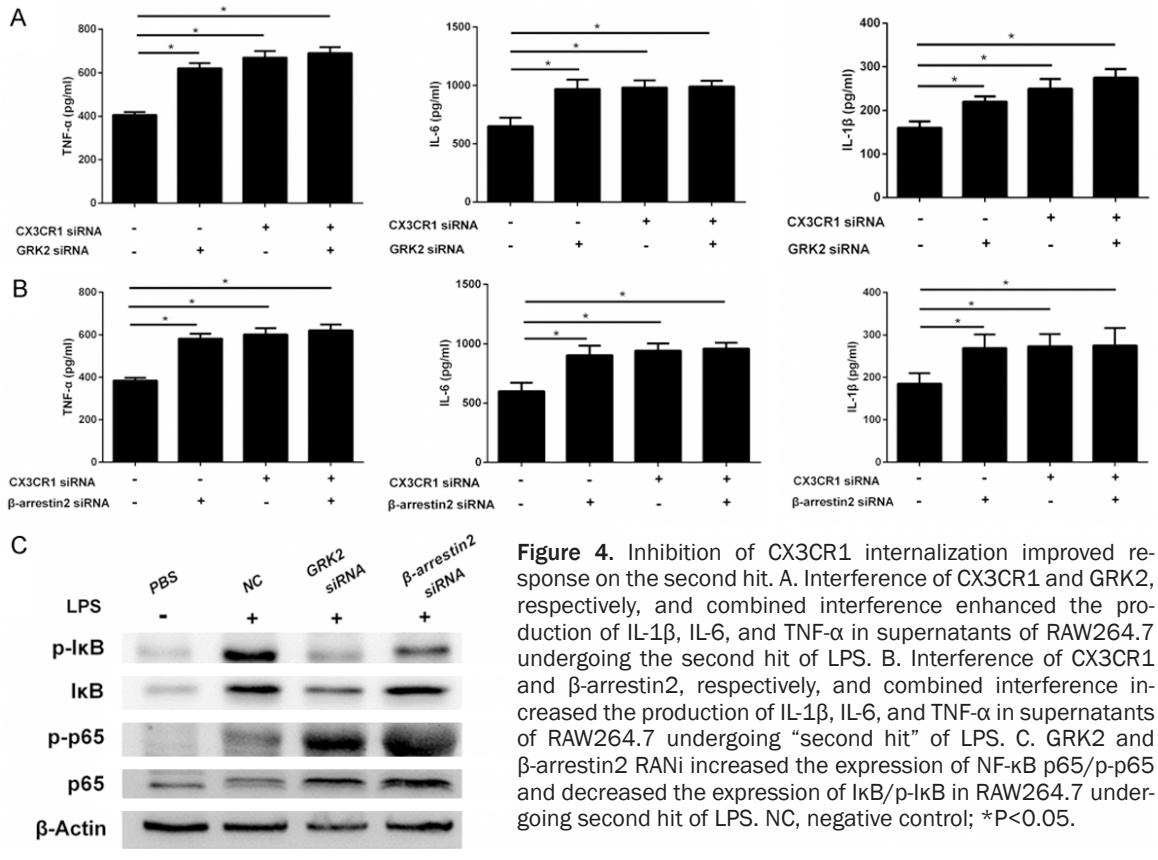

Figure 4. Inhibition of CX3CR1 internalization improved response on the second hit. A. Interference of CX3CR1 and GRK2, respectively, and combined interference enhanced the production of IL-1β, IL-6, and TNF-α in supernatants of RAW264.7 undergoing the second hit of LPS. B. Interference of CX3CR1 and β-arrestin2, respectively, and combined interference increased the production of IL-1β, IL-6, and TNF-α in supernatants of RAW264.7 undergoing "second hit" of LPS. C. GRK2 and β-arrestin2 RANi increased the expression of NF-κB p65/p-p65 and decreased the expression of IκB/p-IκB in RAW264.7 undergoing second hit of LPS. NC, negative control; *P<0.05.

LPS for 24 h resulted in a decreased expression of GRK2 and β-arrestin2 (**Figure 3A**), along with reduced internalization of CX3CR1 (**Figure 3C**). Similar results were observed in the case of TLR4 inhibitor (TAK-242)-treated macrophages (**Figure 3B**, **3C**).

Inhibition of CX3CR1 internalization improved second hit response

Given the non-specificity of GRK2 and β-arrestin2 in CX3CR1 internalization, we employed a combined RNA interference (GRK2 RNAi coupled with CX3CR1 RNAi and β-arrestin2 RNAi coupled with CX3CR1 RNAi) to further substantiate the correlation between CX3CR1 internalization and immunoparalysis. The interference of CX3CR1 and GRK2, respectively and combined interference resulted in an elevated response following the second hit of LPS, but without significant differences between the three groups (**Figure 4A**). Similar results were also observed in β-arrestin2 RNAi and combined interference of both CX3CR1 and β-arrestin2 (**Figure 4B**). Despite the association with other receptors' internalization, additional knockdown of GRK2 and β-arrestin2 did not influence the effects of CX3CR1 interference on responsiveness during immunoparalysis. This indicates that internalization of the other relative receptors may have little effects during the immunoparalysis period. Moreover, the improvement in responsiveness induced by GRK2 and β-arrestin2 interference relied on increased expression of NF-κB p65/p-p65 and decreased expression of IκB/p-IκB (**Figure 4C**).

Discussion

Sepsis is a life-threatening illness, which refers to the systemic inflammatory response following microbial infection [17]. Presently, the mechanism of sepsis-induced immunosuppression is not clear. Previous studies determined the probability of a variety of immune cells involved in late-phase sepsis featured by immunoparalysis. A lack of antigen presenting cells, such as macrophages and dendritic cells, as well as declined expression of inhibitory ligand also contributed to the severe immunoparalysis [18].

Various receptors are involved in the pathophysiology of sepsis. TLR4 has been intensively studied in the past few years and demonstrated as a vital participant in sepsis [19-21]. CX3CR1 has been implicated as a chemotactic factor promoting the release of IL-6, IL-1β, and other proinflammatory cytokines, as well as mediating fever and sickness syndrome in vivo [22, 23]. There is accumulating evidence with respect to the pathophysiological effects of CX3CR1 in the pathogenesis of various inflammatory diseases such as atherosclerosis, allograft rejection, human immunodeficiency virus infection, and rheumatoid arthritis [24]. However, the biological role of CX3CR1 in sepsis-induced immunoparalysis period has not yet been fully elucidated. In the current study, we identified the correlation between CX3CR1 internalization and sepsis-induced immunoparalysis. Both CLP, as the standard murine model of abdominal sepsis and typical LPS-induced cell model were established to discover the effects of CX3CR1 internalization on septic immunoparalysis in our study. The membranous expression of CX3CR1 was significantly decreased in the late phase of sepsis. Moreover, increased internalization of CX3CR1 was observed using immunofluorescence confocal microscopy, which prompted a potential relationship between CX3CR1 internalization and septic immunoparalysis. Internalization plays crucial roles in several aspects of cell biology, ranging from the uptake of nutrients to the regulation of intercellular signaling. An increasing number of studies have indicated the significant interaction between Toll-like receptors and chemokine receptors [25, 26]. We later found that increased internalization of CX3CR1 relied on intact TLR4. Clathrin-dependent endocytic pathways have been demonstrated as one of the major mechanisms of internalization for different chemokine receptors [27, 28]. GRK2 and β-arrestin2 are involved in clathrin-dependent endocytic pathways and play pivotal roles in GPCRs signal transduction, desensitization, and transport. GRK2 promotes the phosphorylation of chemokine receptors, which is critical for the receptor internalization [29-32]. β-arrestin binds with a high affinity to the phosphorylated receptor and further aids in internalization [33, 34]. In addition to the key role in the phosphorylation-dependent GPCR desensitization and endocytosis, GRKs are also able to phosphorylate other membrane receptors [35], and non-receptor substrates [36, 37]. In the phosphorylation-independent processes, GRKs exert an ability to interact with a variety of proteins involved in essential biological pathways [38-40]. Recently, β-arrestin2 has been demonstrated to regulate immune responses through multiple mechanisms, such as regulation of NF-κB [41], ERK1/2 pathway [42], Th1/Th2 imbalance [43], and factor-independent survival of macrophages

[44]. In our study, GRK2 and β-arrestin2 were significantly augmented in the late phase of sepsis. The knockdown of GRK2 and β-arrestin2 resulted in a decreased internalization of CX3CR1 and alleviated immunoparalysis, which was consistent with the augmented expression of NF-κB p65 and decreased expression of IκB. An increased expression of GRK2 and β-arrestin2 were accompanied with the internalization of CX3CR1 during septic immunoparalysis, which may continue to influence NF-κB activation, thereby contributing towards the dysfunction of immune state in the late phase of sepsis.

In conclusion, our study demonstrates that the internalization of CX3CR1 is closely associated with immunoparalysis in the late phase of sepsis. The finding sheds light on the regulatory role of receptor internalization in immunoparalysis stage, which aids in recognizing the potential mechanism of immunoparalysis and providing a novel reference for clinical therapy.

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Disclosure of conflict of interest

None.

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