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Fostamatinib, a Spleen Tyrosine Kinase Inhibitor, Exerts Anti-Inflammatory Activity via Inhibiting STAT1/3 Signaling Pathways

Ran Guo[1], Hanjing Liao[2], Yinuo Meng[3], Xiaoyi Shi[4], Yuting He[4], Zhixiang Zhu[2], Wenzhi Guo[1,4]

[1]Henan Key Laboratory of Digestive Organ Transplantation, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, People's Republic of China; [2]Modern Research Center of Traditional Chinese Medicine, Beijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, Beijing, 100029, People's Republic of China; [3]Department of Faculty of Life Sciences & Medicine, King's College London, London, UK; [4]Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, 450052, People's Republic of China

Correspondence: Wenzhi Guo, Henan Key Laboratory of Digestive Organ Transplantation, the First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Erqi District, Zhengzhou, 450052, People's Republic of China, Email fccguowz@zzu.edu.cn; Zhixiang Zhu, Modern Research Center for Traditional Chinese Medicine, Beijing Research Institute of Chinese Medicine, Beijing University of Chinese Medicine, No. 11 Beisanhuan East Road, Chaoyang District, Beijing, 100029, People's Republic of China, Email zzx@bucm.edu.cn

Background: Fostamatinib is the first spleen tyrosine kinase inhibitor approved for the treatment of chronic adult immune thrombocytopenia via blocking autoantibody-mediated platelet phagocytosis. Nevertheless, the potential of fostamatinib as therapeutic agent against acute inflammatory diseases has not been examined. This study aimed to investigate the effects of fostamatinib on the activation of macrophages and neutrophils and its therapeutic effects on SIRS.

Methods: First, RT-qPCR and ELISA were used to detect the effects of fostamatinib on the expression and secretion of inflammatory factors by peritoneal macrophages (PMs) induced with TLR agonists. The activation and ROS release of neutrophils were detected by flow cytometry. Subsequently, the therapeutic effect of fostamatinib on LPS-induced SIRS in mice was examined. Finally, we also explored the underlying mechanisms of fostamatinib exerting pharmacodynamic effects by analyzing its effects on LPS-induced gene expression profile and the activation of signaling pathways in PMs through transcriptome sequencing and Western blot.

Results: We found that fostamatinib inhibited the expression and secretion of TNF-α, IL-6, CCL2, CCL3, and CXCL10 (*$P < 0.05$) in PMs induced by LPS. Fostamatinib also reduced the activation of neutrophils stimulated by LPS, and suppressed the release of ROS by neutrophils. In SIRS mice, fostamatinib diminished the levels of inflammatory factors, and inhibited the excessive consumption of neutrophils in bone marrow. Transcriptome sequencing results showed that fostamatinib significantly inhibited the transcription of *Cxcl10, Isg20, Mx1, Rsd2*, etc. (*$P < 0.05$) in PMs induced by LPS. Meanwhile, fostamatinib selectively blocked the phosphorylation of STAT1 and STAT3 in PMs induced by LPS and cytokines (IFN-γ and IL-6).

Conclusion: Fostamatinib can significantly inhibit LPS-induced inflammatory response through blocking STAT1/3 signaling pathways and has the potential to be used in the therapy of acute inflammatory diseases, especially SIRS and sepsis, which are resulting from the infection of Gram-negative bacteria.

Keywords: fostamatinib, inflammation, macrophages, neutrophils, STAT1/3 signaling pathways, systemic inflammatory response syndrome

Introduction

Inflammation is an important biological reaction in order to resist microbial infection, eliminate damaged or mutated cells, and maintain the stability of organisms. However, continuous development of inflammation will lead to excessive inflammatory response, and eventually cause severe damage to normal tissues.[1] Acute inflammation is usually initiated by infection, burns, trauma, or intoxication. Chronic inflammation is often secondary to autoimmune diseases and allergic diseases. Sepsis is a kind of acute critical disease involving physiological, pathological, immunological and biochemical abnormalities caused by infection. The main characteristic of sepsis is the occurrence of infection-induced systemic

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inflammatory response syndrome (SIRS), which might result in multiple organs failure and even death. According to epidemiological surveys, there were 48.9 million sepsis cases and 11 million sepsis deaths worldwide in 2017, accounting for about 20% of total global deaths.[2] Sepsis is one of the lethal factors caused by the global COVID-19 epidemic.[3] Currently, the excessive inflammatory response in sepsis is mainly treated with glucocorticoids, such as hydrocortisone and fludrocortisone. However, application of glucocorticoids will suppress entire immune response and cause many other adverse reactions, thus limiting the treatment of sepsis. Over-activation of macrophages and neutrophils plays important roles in the pathogenesis of SIRS and sepsis.[4–6] Inhibiting the over-activation of macrophages or neutrophils with targeted agents probably is an important strategy for treating SIRS and sepsis.

The acute inflammation responses in SIRS and sepsis occur when the pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), mannose receptor, and glucan receptor recognize various pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).[7] Upon stimulation with PAMPs or DAMPs, PRRs on innate immune cells will induce the activation of these cells, thus causing the secretion of inflammatory factors, chemokines, and reactive oxygen species (ROS). Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria and can be recognized by TLR4 on innate immune cells to induce inflammatory response. LPS-induced systemic inflammatory response is similar to the SIRS in sepsis initiated by the infection of Gram-negative bacteria.[8] Probably, specific inhibition of LPS-induced activation of macrophages and neutrophils with targeted agents might have therapeutic effects to SIRS or sepsis initiated by the infection of Gram-negative bacteria.

Spleen tyrosine kinase (SYK) is a non-receptor tyrosine kinase and expressed primarily in hematopoietic cells. SYK binds to immune receptors, such as Fc receptors (FcR), B-cell receptors (BCR), and C-type lectin receptors (CLR). SYK activation regulates multiple biological processes, including secretion of cytokines, antibody-dependent phagocytosis of innate immune cells, IgE-mediated degranulation of mast cells, and platelet aggregation.[9,10] Therefore, SYK inhibitors were used to treat autoimmune diseases by blocking autoantibody-dependent phagocytosis of innate immune cells,[11,12] and allergic diseases via inhibiting IgE-mediated degranulation of mast cells. SYK has also been shown to be activated in macrophages stimulated by TLR agonists. Existing data reveal the constitutive binding of SYK to the cytoplasmic domain of TLR4.[13] This SYK-TLR4 binding increases upon TLR4 dimerization and phosphorylation, and SYK plays a prominent role in TLR4 signaling in response to LPS in macrophages and neutrophils.[14] SYK inhibitors might inhibit the over-activation of macrophages and neutrophils induced by TLR agonists, especially LPS.

Fostamatinib (R788), a SYK inhibitor that is used to treat chronic adult immune thrombocytopenia by inhibiting Fc-activated receptor and B-cell receptor signaling, thereby reducing platelet destruction.[15,16] There are growing evidences that suggest that SYK plays important roles in the activation of macrophages and neutrophils induced by TLR agonists. Consequently, R788 might have the potential to be developed as a therapeutic agent for SIRS or sepsis. In the present study, we examined the anti-inflammatory activity of R788 on macrophages and neutrophils in vitro experiments. Furthermore, its effect on LPS-induced SIRS was studied in vivo experiments. Finally, the underlying mechanisms of R788 exerting anti-inflammatory effects were also explored.

Materials and Methods

Reagents

R788 was purchased from Abmole (Houston, TX, USA). Zymosan, LPS (from Escherichia coli O55:B5), thioglycolate medium, and dihydrorhodamine 123 (DHR 123) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Resiquimod (R848) was purchased from Selleck Chemicals (Houston, TX, USA). CpG ODN-1826 was obtained from Invivogen (San Diego, CA, USA). TRIzol Reagent was from ThermoFisher Scientific (Waltham, MA, USA). TransScript First-Strand cDNA Synthesis Kit and TransStart Green qPCR SuperMix were purchased from TransGen Biotech (Beijing, China). ELISA kits for analyzing TNF-α, IL-6, IL-1β, and IFN-γ were acquired from Mabtech (Stockholm, Sweden). ELISA kit for analyzing CCL2, CCL3, CXCL10, and CRP was purchased from R&D Systems (Minneapolis, MN, USA). Anti-mouse CD11b-APC (M1/70), anti-mouse CD62L-PE (MEL-14), anti-mouse Ly6G-PE/Cyanine7 (1A8), and 7-aminoactinomycin D (7-AAD) were obtained from Biolegend (San Diego, CA, USA). The antibodies to β-actin (#3700), P65 (#8242), phospho-P65 (Ser536) (#3031), P38 (#9212), phospho-P38 (Thr180/Tyr182) (#4511), ERK1/2 (#4695),

phospho-ERK1/2 (Thr202/Tyr204) (#9101), STAT1 (#14994), phospho-STAT1 (Tyr701) (#9167), STAT3 (#12640), and phospho-STAT3 (Tyr705) (#9145) were purchased from Cell Signaling Technology (Beverly, MA, USA). The details of the reagents were listed in Supplementary Table S1.

Animals

Male 8-week-old C57BL/6 mice were purchased from SPF Biotechnology (Beijing, China) and housed at 22 ± 2°C under a 12 h light/dark cycle. Mice were fed standard lab chow with water available *ad libitum*. Animal care followed guidelines issued by the Ethics Committee for Medical Animal Experiments, and the experimental program has been reviewed and approved by the Ethics Committee for Scientific Research and Clinical Trials of the First Affiliated Hospital of Zhengzhou University (2022-KY-0281-002).

Cell Preparation and Culture

PMs were obtained from mouse abdominal cavity according to the method mentioned in the reference.[17] In brief, mice were injected intraperitoneally with 1 mL of 3% thioglycolate medium. After 4 days, mice were killed by cervical dislocation and peritoneal cells were collected by lavage with 5 mL PBS. Red blood cells were removed from peritoneal cells with RBC lysis buffer. PM were further purified by adherence culture in high glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C under 5% CO_2.

Mouse bone marrow (BM) cells were collected by flushing the femur and tibia from mice with PBS, and single-cell suspension was obtained. Red blood cells were depleted by RBC lysis buffer. The remained BM cells were cultured in IMDM supplemented with 2% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Mouse PMs were seeded in 12-well plate (1.5×10^6 cells/well). After incubating overnight, the cells were pretreated with or without 1 μM R788 for 0.5 h and then stimulated with zymosan (10 μg/mL), LPS (1 μg/mL), R848 (10 μM), or CpG ODN-1826 (10 μg/mL) for 6 h. Total RNA was extracted from PMs with TRIzol Reagent according to the manufacturer's instructions. cDNA was synthesized from mRNA with TransScript First-Strand cDNA Synthesis Kit, and then RT-qPCR was conducted with TransStart Green qPCR SuperMix on CFX96 Touch™ PCR System (BioRad, Hercules, CA, USA). The level of mRNA in each sample was analyzed and normalized to the amount of GAPDH mRNA. All primers used in the experiments were synthesized by Sangon Biotech (Shanghai, China) and listed in Table 1.

Table 1 Primer Sequences Used in RT-qPCR Analysis

Gene	Forward (5' -> 3')	Reverse (5' -> 3')
Tnfa	CCCCTTTATTGTCTACTCCT	AAAGCCCATTTGAGTCCTTG
IL6	AGTCGGAGGCTTAATTACACA	TCATACAATCAGAATTGCCAT
IL1b	GCTGCTTCCAAACCTTTGACC	ACAGCCACAATGAGTGATACTGC
Ccl2	ACCTGCTGCTACTCATTCACC	CCATTCCTTCTTGGGGTCAG
Ccl3	ATTCCACGCCAATTCATCGTT	TCTGCCGGTTTCTCTTAGTCA
Cxcl10	TGTTGAGATCATTGCCACGA	AGGAGCCCTTTTAGACCTT
Rasd2	TTGGTGCCTGAATCTAACCAG	TATTGCTTCTTCCACGCCAAC
Cd69	CTTCACATCTGGAGAGAGGGC	ACACAGCCCAAGGGATAGAAA
Isg20	ATTGCCCAGAAGATTGGTGT	CTCGCTGCAGTTCTGTACCAC
Mx1	CCAAGGCATTAATAAACCCTG	TGTCTCCCAATATTCCGTCT
Ifit2	AAGCCTTAAAGAAAGACCCAA	TTTCCAAAGCCTTTCTAAGCA
Cxcl3	TAGATCGGATTCAAGTTACGC	TTGTAACCATTTGGCACGAG
S1pr1	CCCTCTCGGACCTATTAGCAG	GAGAGCCACAAACATACTCCC
GAPDH	GCGACTTCAACAGCAACTCC	CACCCTGTTGCTGTAGCCGT

Enzyme-Linked Immunosorbent Assay (ELISA)

PMs were incubated overnight in 96-well plate (1.5×10^5 cells/well). The cells were pretreated with or without serial dilutions of R788 (0.25, 0.5, and 1 μM) for 0.5 h and then stimulated with LPS (1 μg/mL) for 12 h. The concentrations of TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10 in culture supernatants were determined with ELISA kits. In addition, the concentrations of inflammatory factors in the serum of SIRS mice were also detected as described.

Flow Cytometry

The activation and reactive oxygen species (ROS) release of neutrophils were measured by flow cytometry. In brief, mouse BM cells were seeded in 24-well plate (1×10^6 cells/well). The cells were pretreated with or without serial dilutions of R788 (0.25, 0.5, and 1 μM) for 15 min and then stimulated with LPS (1 μg/mL) for 2 h. The expression of CD62L and CD11b on neutrophils (gated by $Ly6G^+$) were determined by flow cytometry on BD FACSCanto II (BD Biosciences, San Diego, CA). In some experiments, BM cells were pretreated with DHR 123 for 15 min, treated with or without R788 for 15 min, and then stimulated with LPS for 2 h. The fluorescence in neutrophils was analyzed by flow cytometry. Fluorescence intensity indicates the production of ROS in neutrophils.

LPS-Induced SIRS in Mice

Mouse SIRS model was generated by LPS injection. After 1 week's acclimatization, mice were randomly divided into 3 groups (n = 8/group): Control, LPS, and LPS + R788 (30 mg/kg) groups. Mice in R788 group were administered with R788 by gavage. After 1 h, mice in LPS and R788 groups were administered with 5 mg/kg LPS by tail vein injection. Mice were sacrificed 3 h after LPS treatment. Peripheral blood was obtained for routine blood test and analysis of inflammatory factors in serum by ELISA.

In the further experiment, mice (n = 8/group) were randomly divided into 6 groups: Control, R788 (30 mg/kg), LPS, and LPS + R788 (7.5, 15, 30 mg/kg) groups. Mice in R788 and LPS + R788 groups were administered with R788 for 3 days. After 1 h of R788 administration every day, mice in LPS and LPS + R788 groups were given 0.5 mg/kg LPS by intravenous injection. Mice were sacrificed after the last LPS treatment. Peripheral blood was collected for routine blood test and serum was used for ELISA. In addition, BM cells were collected and the percentage of neutrophils in BM cells was analyzed by flow cytometry.

Transcriptome Sequencing and Validation

PMs were seeded in 6-well plate (2.5×10^6 cells/well) overnight. The cells were pretreated with or without R788 (1 μM) for 0.5 h and then stimulated with LPS (1 μg/mL) for 6 h. The cells were then collected and mRNA was extracted. cDNA libraries were constructed by using Illumina Truseq kit and transcriptome sequencing was conducted on Illumina's HiSequation 2000. The sequencing results were aligned with the mouse GRCm38 genome by HiSAT, and the differential gene expression was analyzed by DESeq2. Differentially expressed genes (DEGs) were presented in the form of Venn diagram and heatmap. Meanwhile, RT-qPCR was used to verify several significant upregulated and downregulated genes induced by R788. The primers were also listed in Table 1.

Immunoblotting Analysis

PMs were seeded in 6-well plate (2.5×10^6 cells/well) overnight. The cells were treated with or without serial dilutions of R788 and then stimulated with LPS (1 μg/mL), IFN-γ (10 ng/mL), and IL-6 (10 ng/mL) for 0.5 or 3 h. The cells were then collected, and total cell proteins were extracted with RIPA lysis buffer. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membrane was blocked in 5% skim milk for 1 h and incubated overnight at 4°C with monoclonal antibodies against β-actin, P38, ERK1/2, P65, STAT1, STAT3, and their phosphorylated forms. The membranes were then washed and incubated with appropriate HRP-conjugated secondary antibodies for 2 h at room temperature. After washing again, protein bands were visualized with ECL reagents on Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA) and analyzed with Quantity One software.

Statistical Analyses

All experiments were repeated at least 3 times. The quantitative data were expressed as the mean ± SEM. For statistical comparison, results were analyzed by Student's *t* test or one way ANOVA followed by Dunnett's post hoc test. A *P* value less than 0.05 was considered statistically significant.

Results

Effects of R788 on the mRNA Transcription of Inflammatory Factors and Chemokines in PMs Induced by TLR Agonists

To observe the anti-inflammatory activity, the effects of R788 on the mRNA transcription of inflammatory factors and chemokines in PMs induced by different TLR agonists were first investigated. The results (Figure 1) showed that compared with vehicle group, TLR agonists induced the mRNA transcription of inflammatory factors and chemokines in PMs to varying degrees. R788 treatment inhibited the mRNA transcription of TNF-α, IL-6, CCL2, CCL3, and CXCL10

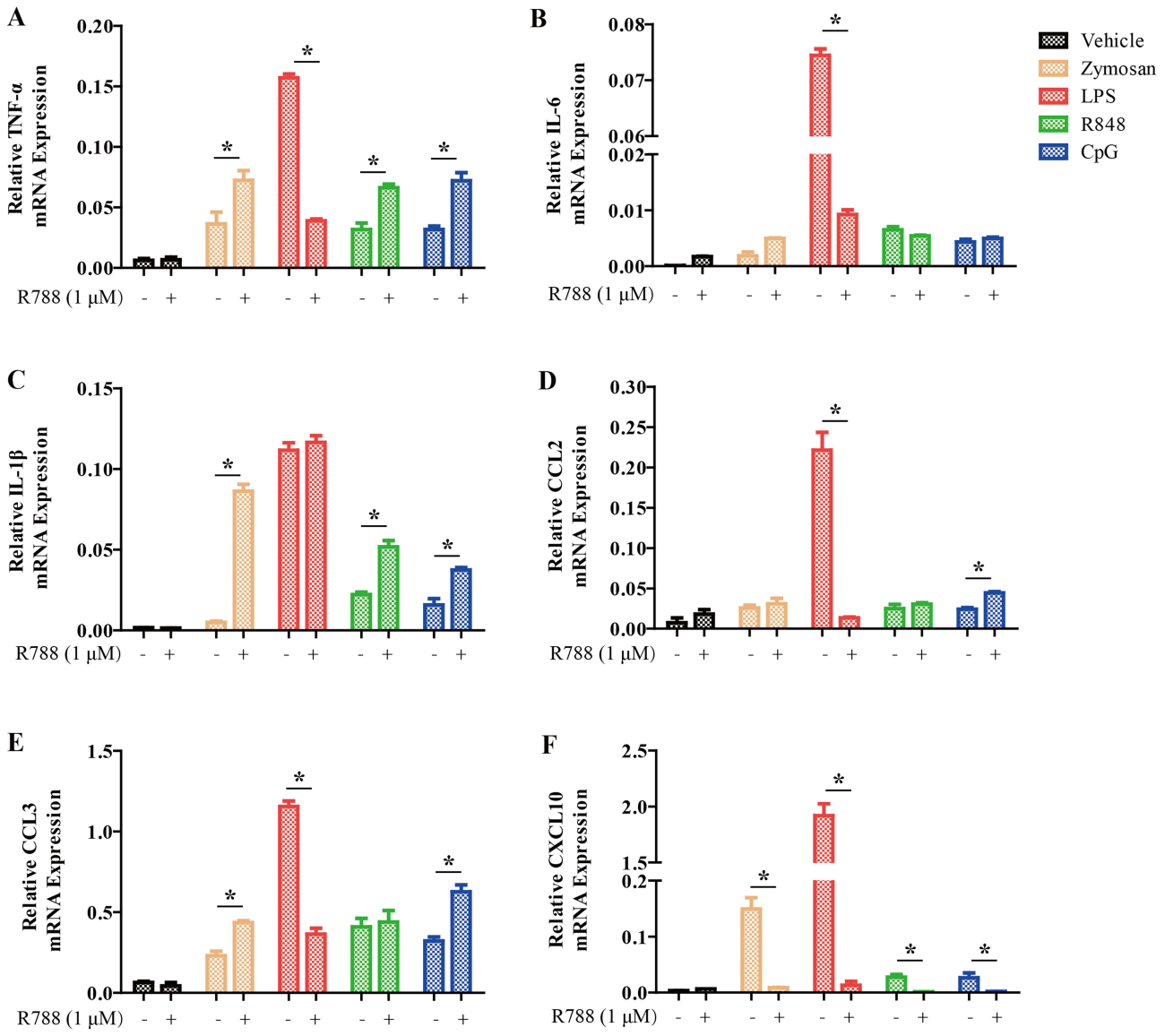

Figure 1 Effects of R788 on the mRNA transcription of inflammatory factors and chemokines in PMs induced by TLR agonists. (**A-F**) The mRNA transcription levels of TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10 in PMs induced by TLR agonists were determined by RT-qPCR. The data are expressed as the means ± SEM of values from three independent experiments. * $P < 0.05$ vs Agonists.

in PMs induced by LPS (*$P < 0.05$), and also suppressed the mRNA transcription of CXCL10 in PMs induced by zymosan, R848, and CpG (*$P < 0.05$). However, R788 significantly promoted the mRNA transcription of TNF-α and IL-1β in PMs induced by zymosan, R848, and CpG (*$P < 0.05$). In addition, R788 also slightly increased the mRNA transcription of CCL2 induced by CpG and CCL3 induced by zymosan and CpG (*$P < 0.05$). The data suggest that R788 selectively inhibits the mRNA transcription of multiple inflammatory factors and chemokines in PMs induced by LPS, and strongly suppresses the mRNA transcription of CXCL10 induced by multiple TLR agonists.

R788 Inhibited the mRNA Transcription of Inflammatory Factors and Chemokines in LPS-Induced PMs

To further confirm the inhibitory effect of R788 on the LPS-induced inflammatory response, different concentrations of R788 were added to detect its effects on the mRNA transcription of related inflammatory mediators. The results (Figure 2) showed that LPS-induced mRNA transcription of inflammatory factors and chemokines, such as TNF-α, IL-6, CCL2, CCL3, and CXCL10, were significantly suppressed by R788 in a dose-dependent manner (*$P < 0.05$).

R788 Suppressed the Secretion of Inflammatory Factors and Chemokines by LPS-Induced PMs

After the inhibitory effects of R788 on the mRNA transcription of inflammatory factors and chemokines in PMs induced by LPS were discovered, we further investigated the effects of R788 on the secretion of these factors. As shown in Figure 3, compared with the vehicle group, LPS intensively induced the secretion of TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10 by PMs. R788 treatment significantly decreased the secretion of TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10 by PMs stimulated with LPS in a dose-dependent manner (*$P < 0.05$).

Inhibitory Effects of R788 on the Activation of Neutrophils Induced by LPS

Neutrophils are important executor of the body's non-specific defense response and play important roles in inflammatory response. However, excessive activation of neutrophils and continuous production of ROS can cause serious tissue damage. The upregulation of CD11b and downregulation of CD62L on neutrophils promote neutrophils activation and migration into inflammatory sites. In order to analyze the regulatory effects of R788 on the activation of neutrophils, we determined its effects on the expression of CD11b and CD62L and the production of ROS by flow cytometry. The results (Figure 4A-D) showed that R788 intensively ameliorated CD11b upregulation and CD62L shedding induced by LPS (*$P < 0.05$). Furthermore, R788 significantly reduced ROS release of neutrophils induced by LPS (Figure 4E and F, *$P < 0.05$).

The Anti-Inflammatory Activity of R788 on LPS-Induced SIRS in Mice

Due to the inhibitory effects of R788 on the mRNA transcription and secretion of inflammatory mediators by PMs and the activation and ROS production of neutrophils in vitro experiments, it was hypothesized that R788 can exert therapeutic effects to acute inflammatory diseases. Therefore, we constructed SIRS model by tail vein injection of LPS, and the anti-inflammatory effects of R788 were investigated. The ELISA results showed that treatment with 5 mg/kg of LPS induced acute inflammatory response in mice and the concentrations of inflammatory factors (TNF-α, IFN-γ, and IL-6), chemokines (CCL2), and acute phase proteins (CRP) in the serum significantly elevated. However, R788 did not reduce the levels of these cytokines (Supplementary Figure S1), which was not consistent with the results in vitro experiments. This may be attributed to the relatively severe acute inflammatory response induced by high-dose LPS and the short administration time of R788.

Therefore, we further optimized the in vivo experiments. A lower dose of LPS (0.5 mg/kg) was injected to mice for 3 days, and serial doses of R788 (7.5, 15, and 30 mg/kg) were administered by gavage 1 hour before every LPS injection. The concentrations of inflammatory factors in the serum were then analyzed. The results showed that, compared with the control group, low-dose and continuous LPS administration still increased the concentrations of inflammatory factors in serum, and R788 significantly reduced the concentrations of TNF-α, IFN-γ, IL-6, CCL2, CXCL10, and CRP (Figure 5D-I, *$P < 0.05$). In addition, the number of white blood cells in the peripheral blood decreased due to the consumption of inflammation, which

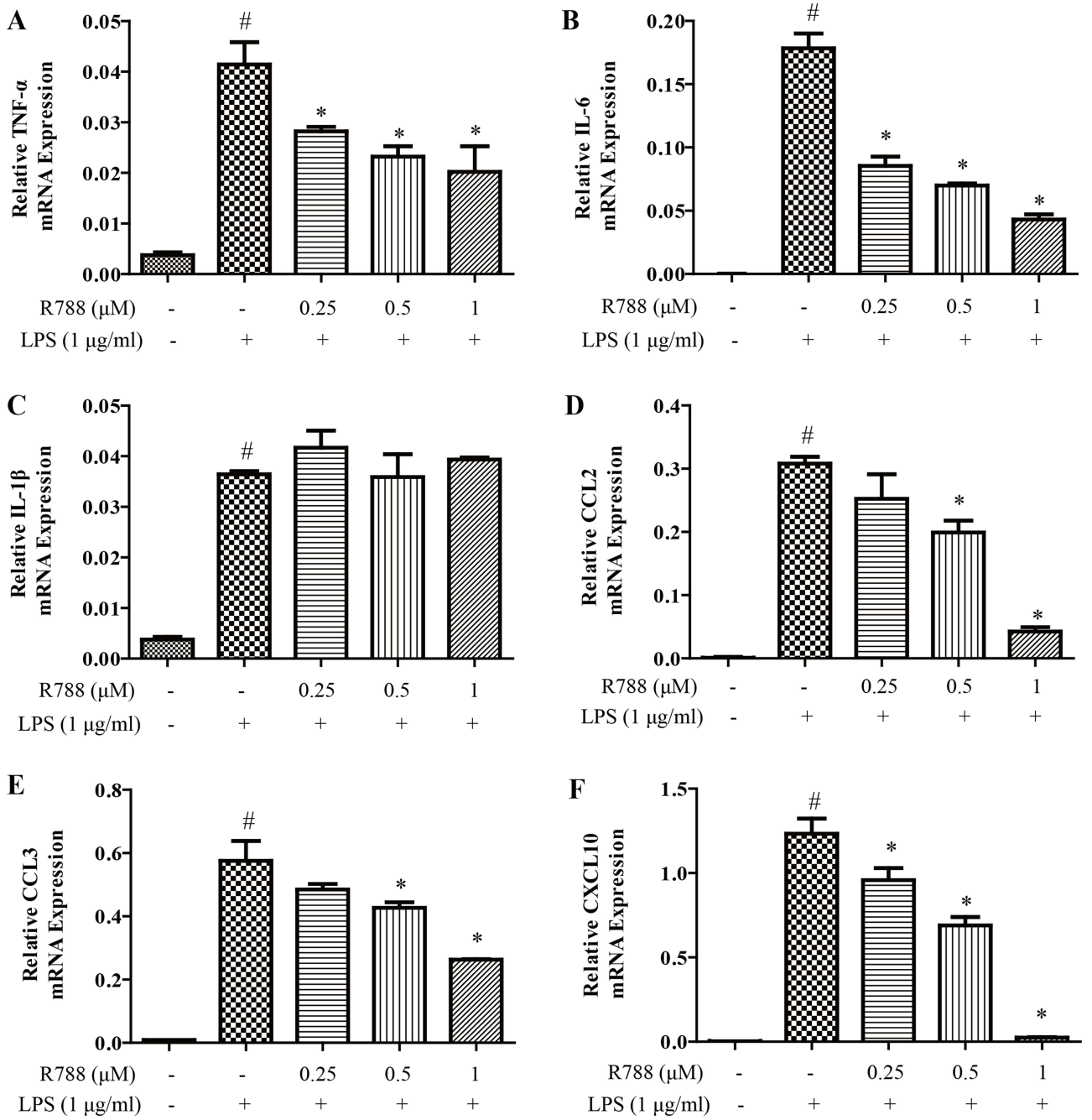

Figure 2 R788 inhibited the mRNA transcription of inflammatory factors and chemokines in LPS-induced PMs. (**A-F**) The mRNA transcription levels of inflammatory cytokines and chemokines in LPS-induced PMs, including TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10, were determined by RT-qPCR. The data are expressed as the means ± SEM of values from three independent experiments. # $P < 0.05$ vs Vehicle; * $P < 0.05$ vs LPS.

was alleviated by R788 (Figure 5A-C). Meanwhile, the flow cytometry analysis (Figure 5J-L) showed that 15 mg/kg and 30 mg/kg R788 also partially reversed the reduction of neutrophils in bone marrow induced by acute inflammation response (*$P < 0.05$). Taken together, the data demonstrate that R788 may be an effective drug for the treatment of acute inflammation such as SIRS.

The Effect of R788 on Gene Expression Profile of LPS-Induced PMs

Differentially expressed genes between groups were identified from transcriptome sequencing data. Compared with control group (C), R788 (R) induced upregulation of 103 genes and downregulation of 704 genes in PMs, and LPS (L) caused

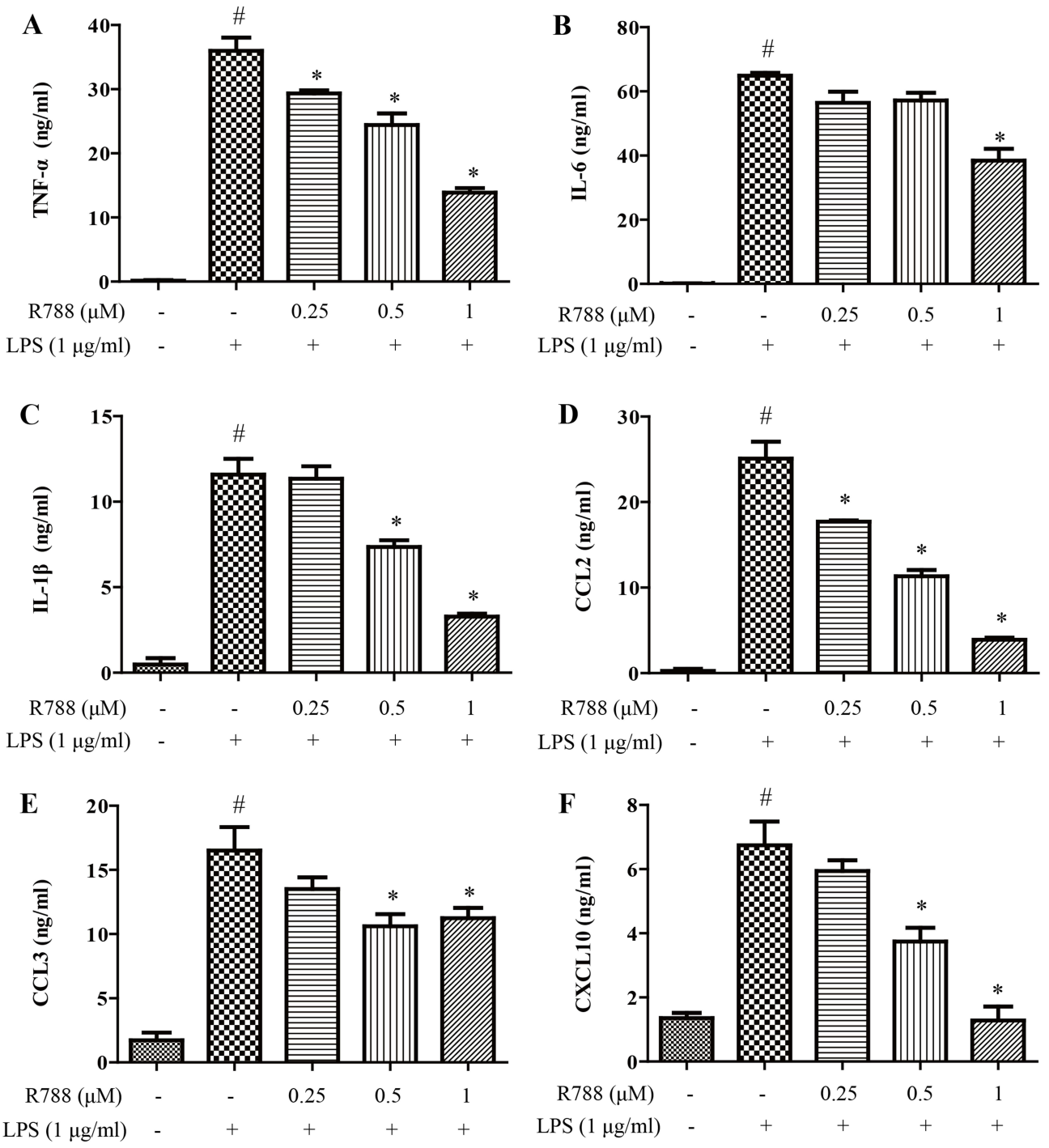

Figure 3 R788 suppressed the secretion of inflammatory factors and chemokines by LPS-induced PMs. (**A-F**) The concentrations of TNF-α, IL-6, IL-1β, CCL2, CCL3, and CXCL10 in the LPS-induced PMs supernatant were detected by ELISA. The data are expressed as the means ± SEM of values from three independent experiments. # $P < 0.05$ vs Vehicle; * $P < 0.05$ vs LPS.

upregulation of 1644 genes and downregulation of 1450 genes. Meanwhile, compared with LPS group, combination of LPS and R788 (LR) increased the expression of 1758 genes and reduced the expression of 2247 genes (Figure 6A and B). We further screened several genes that were significantly regulated by R788. The top up- or down-regulated genes upon R788 treatment were shown in Figure 6C and D respectively. Among these differentially expressed genes, R788 significantly inhibited LPS-induced transcription of inflammation-related genes, such as interferon-inducible genes *Ifit1* (6.71-fold), *Ifi208* (6.26-fold), *Ifit2* (5.58-fold), *Ifi203* (3.94-fold), interferon-stimulated gene *Isg20* (4.63-fold), chemokine *Cxcl10* (6.04-fold),

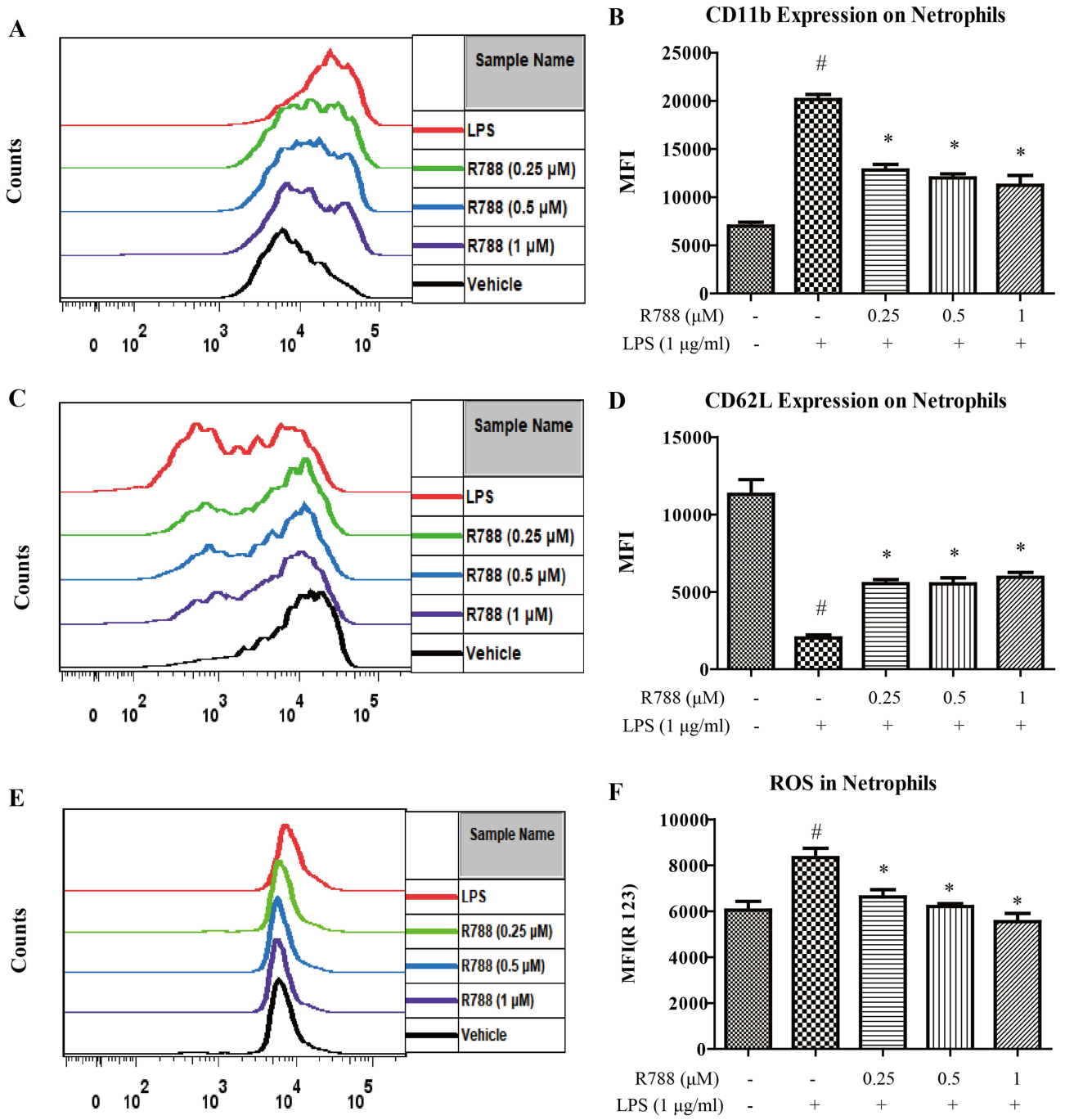

Figure 4 Inhibitory effects of R788 on the activation of neutrophils and ROS release induced by LPS. (**A-D**) The expression of CD11b and CD62L on neutrophils were determined by flow cytometry. (**E** and **F**) ROS production in neutrophils was quantitated by fluorescence analysis of DHR 123 with flow cytometry. The data are expressed as the means ± SEM of values from three independent experiments. # $P < 0.05$ vs Vehicle; * $P < 0.05$ vs LPS.

Cxcl11 (8.71-fold), *Ccl12* (4.11-fold), inflammatory factor receptor *Il27* (4.72-fold), *Il19* (4.74-fold), myxovirus-resistant genes *Mx1* (4.97-fold), *Mx2* (4.74-fold), and Schlafen genes *Slfn4* (5.17-fold), *Slfn9* (5.08-fold). In addition, to verify the reliability of transcriptome sequencing data, the transcription levels of six down-regulated genes (*Rasd2, Cxcl10, Cd69, Isg20, Mx1*, and *Ifit2*) and two up-regulated genes (*Cxcl3* and *S1pr1*) by R788 treatment were validated by RT-qPCR. The results showed that the trend of gene transcription was consistent with the sequencing results (Figure 6E and F). Collectively, the data suggest that R788 can significantly alter the gene expression profile of LPS-induced PMs, especially inhibiting the transcription of many interferon-inducible genes.

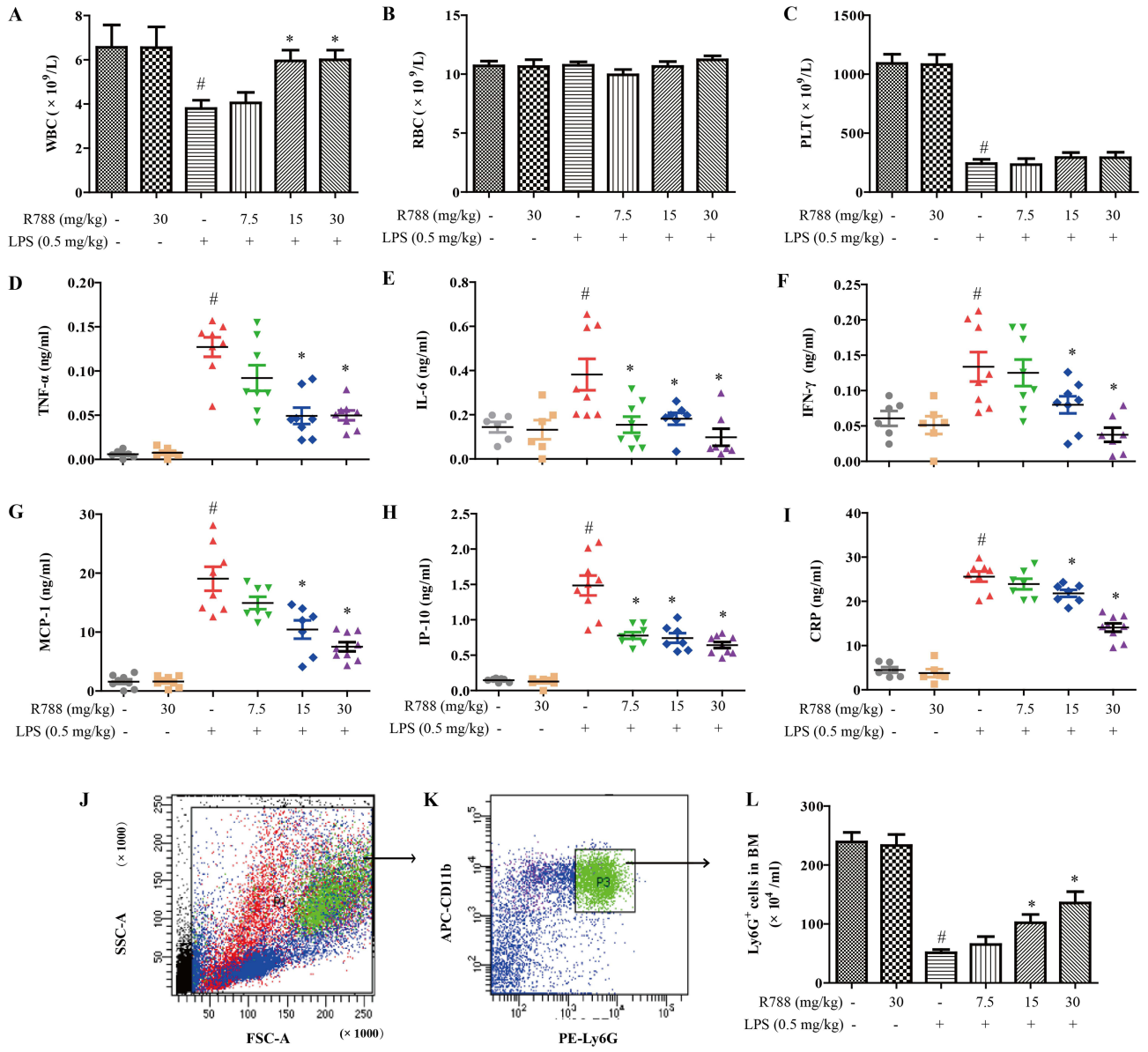

Figure 5 Anti-inflammatory activity of R788 on SIRS induced by consecutive administration of low-dose LPS. (**A-C**) The counts of WBC, RBC, and PLT in the peripheral blood were analyzed by blood routine determination. (**D-I**) The concentrations of TNF-α, IL-6, IFN-γ, CCL2, CXCL10, and CRP in serum were detected by ELISA. (**J-L**) The percentages and numbers of neutrophils in bone marrow were determined by flow cytometry. The data are expressed as the means ± SEM of values from a representative experiment performed with 8 mice per group. # $P < 0.05$ vs Control group; * $P < 0.05$ vs Model group.

Effects of R788 on the Activation of Signaling Pathways in PMs Stimulated by LPS or Cytokines

The activation of NF-κB, MAPK, and STAT1/3 signaling pathways plays critical roles in the function regulation of macrophages and neutrophils. To reveal the mechanisms of R788 exerting anti-inflammatory activity, we evaluated the effects of R788 on the phosphorylation of key components in these signaling pathways in PMs stimulated by LPS. The results (Figure 7A-D) showed that R788 significantly inhibited the phosphorylation of STAT1 (*$P < 0.05$) and STAT3 (*$P < 0.05$) in PMs induced by LPS but did not alter the phosphorylation of P65, P38, and ERK1/2. Since the phosphorylation of STAT1 and STAT3 in PMs is mainly stimulated by interferons and IL-6, respectively, we further examined the activation of STAT1/3 in PMs directly induced by IFN-γ and IL-6 in the presence of R788. The results (Figure 7E-G) showed that the phosphorylation of STAT1 (*$P < 0.05$) and STAT3 (*$P < 0.05$) in PMs stimulated by IFN-

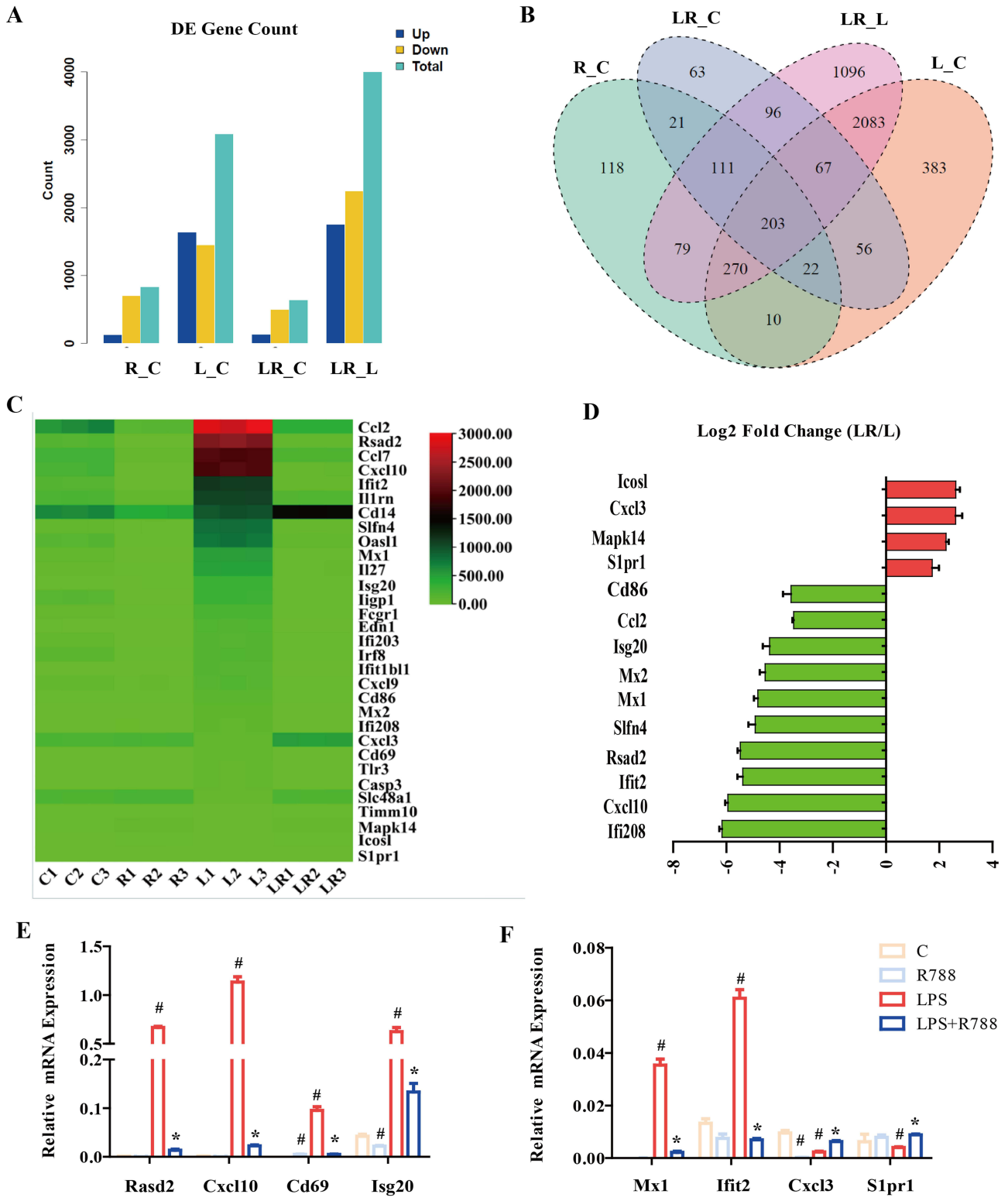

Figure 6 The effect of R788 on the gene expression profile of LPS-induced PMs. (**A**) Statistical analysis of gene expression variation among four groups was performed. (**B** and **C**) Venn diagram and heatmap of showing gene expression differences were made. (**D**) A summary of top ten down-regulated and four up-regulated genes in PMs following R788 treatment compared with the LPS was also displayed. (**E** and **F**) Transcription levels of eight genes were validated by RT-qPCR. # $P < 0.05$ vs Vehicle; * $P < 0.05$ vs LPS.

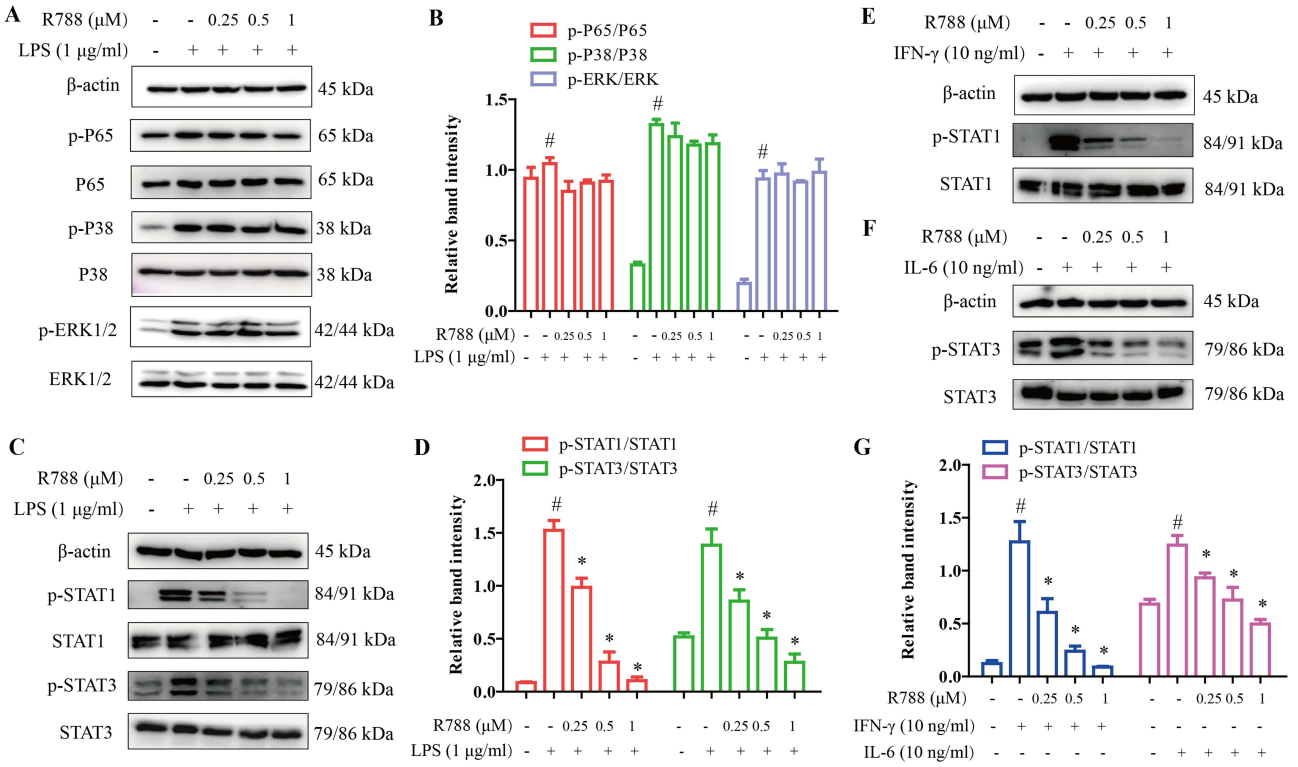

Figure 7 Effects of R788 on the activation of signaling pathways in PMs stimulated by LPS or cytokines. (**A** and **B**) The levels of total proteins and their phosphorylated forms of P65, P38, and ERK1/2 were detected in PMs stimulated with LPS (1 μg/mL) for 30 min. (**C** and **D**) STAT1, STAT3, and their phosphorylated forms in PMs stimulated with LPS (1 μg/mL) for 3 h were detected. (**E-G**) STAT1, STAT3, and their phosphorylated forms in PMs induced with IFN-γ (10 ng/mL) or IL-6 (10 ng/mL) for 30 min were detected. The level of each protein was quantified using Quantity One software. The data are expressed as the means ± SEM of values from three independent experiments. # $P < 0.05$ vs Vehicle; * $P < 0.05$ vs Stimuli.

γ and IL-6 were also significantly inhibited by R788. The results suggest that R788 might exert anti-inflammatory effect via directly inhibiting the activation of STAT1/3 signaling pathways induced by cytokines.

Discussion

Fostamatinib is a specific SYK inhibitor, which was approved by the FDA in 2018 for the treatment of chronic adult immune thrombocytopenia.[18] In recent years, its inhibitory activities in autoimmune diseases and chronic inflammation have also been gradually revealed and reported. It was demonstrated to have activities of treating rheumatoid arthritis by alleviating the degree of swelling and inflammation of the joints.[19] Fostamatinib could also decrease the severity of colonic mucosal damage in a rodent model of colitis.[20] In addition, fostamatinib reduced the expression of mast cell-associated genes, activation of signaling pathways in mast cells and the number of tryptase/chymase positive mast cells in lesional hidradenitis suppurativa tissue.[21] These reports demonstrate the great potential of fostamatinib to resist inflammatory injuries. In order to develop new application for fostamatinib in the therapy of acute inflammatory diseases, we investigated the effect of fostamatinib on excessive activation of macrophages and neutrophils induced by LPS, and evaluated its therapeutic effect to LPS-induced SIRS in mice.

Macrophages are the main inflammatory effector cells in the body, which can undergo pro-inflammatory differentiation and then secret inflammatory mediators, such as NO, TNF-α, IL-6, CCL2 under the stimulation of LPS. Studies have shown that the infiltration and activation of macrophages play an important role in sepsis, acute lung injury, peritonitis and other inflammatory diseases.[22–24] TLRs are considered to be the most important PAMPs, which are widely distributed in immune cells, such as macrophages, neutrophils, and dendritic cells.[25,26] So we first examined the effect of fostamatinib on activated macrophages induced by different TLR agonists, the results showed that fostamatinib selectively inhibited the mRNA transcription of TNF-α, IL-6, CCL2, and CCL3 in PMs induced by LPS. Interestingly, fostamatinib not only exerted strongest inhibition on the expression of CXCL10 induced by LPS but also significantly

inhibited the expression of CXCL10 stimulated by other agonists. Some studies showed that the expression of CXCL10 in PMs is highly dependent on the activation of STAT1 signaling pathway.[27,28] Therefore, the strongly inhibitory effects of fostamatinib on the expression of CXCL10 in PMs induced by multiple TLR agonists preliminarily indicate its potential mechanisms of exerting anti-inflammatory activity may associate with STAT signaling pathway.

Infection, burns, wounds and other conditions can lead to acute inflammation, resulting in the release of a large number of inflammatory factors, causing SIRS, and then developing into multiple organ failure and eventually leading to death.[29] In this study, SIRS model was first constructed by intravenous injection of 5 mg/kg of LPS and inflammatory cytokines in the serum were measured. The results showed that 30 mg/kg of fostamatinib had no effect on LPS-induced elevation of proinflammatory cytokines and chemokines in the serum. We speculated that it was caused by given excessive dose of LPS and the short administration time of fostamatinib. After all, fostamatinib exerted significant inhibitory effects on overactivated macrophages and neutrophils in vitro experiments. In addition, the infection of bacteria or viruses in the body usually lasts for more than 24 h. If we choose a low-dose of LPS to construct SIRS model, it may be more consistent with the actual clinical situation of SIRS. Subsequently, the mice were administered with 0.5 mg/kg of LPS for three consecutive days, and different doses of fostamatinib were given before LPS injection in every day. The results showed that 15 and 30 mg/kg of fostamatinib significantly reduced the concentrations of inflammatory factors (TNF-α, IFN-γ, and IL-6), chemokines (CCL2 and CXCL10), and acute phase protein (CRP) in the serum, and increased the number of white blood cells in peripheral blood and the percentage of neutrophils in bone marrow, which indicated that fostamatinib reduced the consumption of white blood cells and the mobilization of bone marrow cells during excessive inflammatory response. The results suggest that fostamatinib can exert therapeutic effects on SIRS.

In order to comprehensively reveal the regulatory effects of fostamatinib on the activation of macrophages and its anti-inflammatory mechanisms, we performed transcriptome analysis. The results showed that compared with LPS group, combination of LPS and R788 increased the expression of 1758 genes and reduced the expression of 2247 genes. Especially, fostamatinib significantly inhibited the transcription of multiple interferon-inducible genes. GO analysis showed that fostamatinib was mainly involved in biological processes such as response to stimuli, response to viruses, immune system processes, and cytokine-mediated signaling pathways. KEGG analysis showed that fostamatinib mainly affected JAK-STAT signaling pathways, TNF signaling pathways, Epstein–Barr virus infection, and cytokine–cytokine receptor interaction. As same as CXCL10, the mRNA transcription of these interferon-inducible genes is also highly dependent on the activation of STAT1 signaling pathway. Therefore, fostamatinib might exert pharmacological activities mainly through inhibiting STAT signaling pathways.

In immunoblotting analysis, fostamatinib did not inhibit the activation of NF-κB and MAPK signaling pathways, but had strong inhibitory effects on LPS-induced activation of STAT signaling pathways. The activation of STAT signaling pathways in PMs induced by LPS is resulting from the signal transduction of interferon regulatory factors (IRFs)-associated signaling pathways or stimulation of cytokines secreted by PMs.[30] Thus, the inhibitory effects of fostamatinib on STAT signaling pathways in PMs induced by LPS might be resulting from its inhibition on the activation of upstream signaling pathways of IRFs induced by LPS or JAKs signaling pathways stimulated by cytokines. IFN-γ has strong immunomodulatory function mainly through activating JAK-STAT1 signaling pathway. Activated STAT1 forms a dimer and activates the expression of genes containing gamma interferon-activated sequence (GAS) elements.[31,32] IL-6 is the main activation signal of STAT3, which mediates the formation of IL-6-gp130 and IL-6-IL-6R complexes to activate the downstream JAK-STAT3 signaling pathway, which is of great significance in inflammatory response and immunity.[33,34] In the present study, phosphorylation of STAT1 and STAT3 in macrophages stimulated by IFN-γ and IL-6 were also significantly inhibited by fostamatinib. These findings suggest that fostamatinib, a SYK inhibitor, may act in a manner similar to JAK inhibitors in the inflammatory response, directly affecting the activation of STAT signaling pathways. Zhang et al[35] reported that fostamatinib did not affect the phosphorylation of SYK but inhibited the phosphorylation of JAK2 in Ramos cells, which indicates that SYK might be a kinase upstream of JAKs and fostamatinib blocks the activation of STAT1/3 by inhibiting the phosphorylation of JAKs in PMs. Collectively, fostamatinib exerts inhibitory effects on LPS-induced inflammatory effects mainly through blocking STAT signaling pathways.

In conclusion, fostamatinib can markedly attenuate the expression and secretion of LPS-induced inflammatory cytokines in macrophages, reduce activation of neutrophils and release of ROS, and also exert therapeutic effects on

LPS-induced SIRS. The anti-inflammatory mechanism of fostamatinib may be via blocking STAT1/3 signaling pathways. Fostamatinib has great potential to be used in the therapy of acute inflammatory diseases, especially SIRS or sepsis resulting from the infection of Gram-negative bacteria.

Of course, this study also has certain limitations. First, our experimental data are derived from rodent experiments, so it is necessary to further validate the results in human. Second, whether fostamatinib can be used in combination with antimicrobial drugs for sepsis therapy is also worth further study.

Data Sharing Statement

The transcriptome sequencing raw data for this study has been uploaded in Mendeley at https://data.mendeley.com/datasets/x6nnxygn48/1.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published.

Disclosure

The authors declare that they have no conflicts of interest.

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