

Original Article

Ulinastatin reduces LPS-induced THP-1 macrophage M1-like characteristics

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Abstract: *Objective:* Ulinastatin could inhibit inflammatory responses, and might be used for treating sepsis. Macrophages can be polarized to type 1 macrophages (M1) when stimulated by lipopolysaccharide and proinflammatory cytokines. M1 participates in proinflammatory responses and is vital in host defense against bacterial and viral infections. However, the regulation of macrophage polarization by ulinastatin is not clear yet. *Methods:* Ulinastatin (2000 U/mL) was used to treat lipopolysaccharide (LPS) (100 µg/mL)-induced THP-1 macrophages. Proinflammatory cytokines were tested by real-time polymerase chain reaction and enzyme-linked immunosorbent assay. Cell surface markers were detected by flow cytometry. Total and phosphorylated p65, STAT (signal transducers and activators of transcription) 1, and STAT3 were detected by Western blotting. *Results:* Ulinastatin downregulated the secretion of proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor alpha, IL-6, and IL-12 by LPS-induced THP-1 macrophages ($P < 0.01$). The mRNA expression of these cytokines was also significantly reduced by ulinastatin. Ulinastatin also upregulated HLA-DR (human leukocyte Antigen DR) but downregulated CD163. The phosphorylation of p65, STAT1, and STAT3 in THP-1 macrophages decreased after ulinastatin treatment. *Conclusion:* Ulinastatin reduced THP1 macrophage M1-like characteristics after LPS treatment, suggesting that the therapeutic effect of ulinastatin on various diseases might be due to the regulation of macrophage polarization.

Keywords: Lipopolysaccharide, macrophage polarization, ulinastatin

Introduction

Ulinastatin, a trypsin inhibitor, can be isolated from adult male urine extract. It is an important drug for treating acute pancreatitis [1-3]. Many similarities exist between pancreatitis and sepsis. Previous studies suggested that ulinastatin might be useful in treating sepsis [4, 5]. However, how ulinastatin exerts its effect is still not well known. The regulation of inflammation by ulinastatin might be vital in regulating inflammation in sepsis.

Macrophages can be activated by bacterial endotoxins such as lipopolysaccharides (LPS), which are crucial in diseases such as sepsis [6]. LPS could activate nuclear factor (NF)-κB, STAT1, and STAT3 pathways, producing proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6. Proinflammatory cytokines secreted by macrophages are important in the development of sepsis [7].

Macrophages can be polarized to type 1 macrophages (M1) when stimulated by lipopolysaccharide and proinflammatory cytokines. M1 participate in proinflammatory responses and are significant in host defense against bacterial and viral infections, while type 2 macrophages (M2) are associated with anti-inflammatory response, parasite infection, tissue remodeling, fibrosis, and tumor progression [8, 9]. The M1 polarization of macrophages is important in sepsis for its role in bacterial clearance. However, excessive activation of macrophages leads to the damage of important organs such as heart and lung [7]. Therefore, the regulation of macrophage inflammatory response and M1 polarization are of great importance in sepsis treatment.

Previous studies showed that ulinastatin could inhibit the inflammatory responses and reduced LPS-induced lung damage [10]. Ulinastatin inhibited the NF-κB signaling pathway and LPS-

Regulatory effects of ulinastatin on macrophages

induced inflammation, reducing the serum concentration of IL-1 β and MCP (monocyte chemoattractant protein) -1 [11, 12]. The regulation of secretion of inflammatory cytokines from granulocytes and macrophages by ulinastatin might be one of the important mechanisms underlying its protective effects against sepsis.

However, the regulation of macrophage polarization by ulinastatin is not clear yet. In this study, the LPS-induced THP1 macrophages were used as a cell model. The study explored the regulatory effects of ulinastatin on macrophages. This study provided further basis for applying ulinastatin against inflammatory diseases.

Materials and methods

THP-1 macrophage culture

The human THP-1 cell line, an acute monocytic leukemia cell line (ATCC TIB-202), was cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μ M) at 37°C in a 5% CO₂ incubator. Further, 10⁶ cells per mL were seeded into 24-well plates to induce the differentiation of THP-1 cells into macrophages, with 200 nM PMA (phorbol 12-myristate 13-acetate) for 48 h. LPS (100 μ g/mL) were used to treat THP1 macrophages. Also, Ulinastatin (2000 U/mL) was used.

Enzyme-linked immunosorbent assay

Cell-free supernatants were collected and kept in the refrigerator at -80°C. Enzyme-linked immunosorbent assay (ELISA) kits for IL-1 β , TNF- α , IL-6, and IL-12 were purchased from Thermo Scientific (San Jose, CA, USA). All the ELISA assay kits were used following the manufacturer's protocol.

RNA isolation, reverse transcription, and real-time polymerase chain reaction

Total RNA was extracted using TRIzol (Thermo Scientific). cDNA synthesis and real-time polymerase chain reaction (PCR) analyses were performed using a Power SYBR Green RNA-to-CT 1-Step Kit (Thermo Scientific) on an Applied Biosystems (Foster City, CA, USA) Real-Time PCR System. The level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression

served as control. The primers used were as follows. IL1 β , forward primer, 5'-ATGGACAAG-CTGAGGAAGATG-3' and reverse primer, 5'-CC-CATGTGTGCGAAGAAGATAGG-3'; TNF- α , forward primer, 5'-CCAGGGACCTCTCTAATCA-3' and reverse primer, 5'-TCAGCTTGAGGGTTTGCTAC-3'; IL-12p40, forward primer, 5'-ACCAGAGCAG-TGAGGTCTTA-3' and reverse primer, 5'-CTCCT-TTGTGACAGGTGACTG-3'; IL-6, forward primer, 5'-GGAGACTTGCCTGGTGA AAA-3' and reverse primer, 5'-CTGGCTTGTTCCCTACTACTC-3'; GAPDH forward primer, 5'-GGTGTGAACCATGAGAA-GTATGA-3' and reverse primer, 5'-GAGTCCTTC-CACGATACCAAAG -3'.

Flow cytometric analysis

THP-1 cells were labeled with PE (phycoerythrin)-conjugated HLA-DR or CD163. Nonspecific isotype-matched antibodies served as controls. All the antibodies were purchased from BD Pharmingen (San Jose, CA, USA). The cells were analyzed by flow cytometry in a FACSCalibur cytometer (BD).

Western blotting

After indicated treatment, the cells were harvested and lysed in RIPA buffer with a protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). The protein concentration of lysates was quantified by the Bradford protein assay (Pierce, Rockford, IL). Equal amounts of protein (50 μ g) were electrophoresed in 5% or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were blocked for 1 h with 5% bovine serum albumin (BSA) in phosphate-buffered saline and incubated with primary antibodies diluted 1:1000 in 5% BSA at 4°C overnight. After washing, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Beyotime, Shanghai, China) diluted 1:4000 in 5% BSA. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's instructions. When necessary, the membranes were stripped by incubation in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50°C. After washing, the membranes were re probed with other antibodies. GAPDH was used as a control.

Regulatory effects of ulinastatin on macrophages

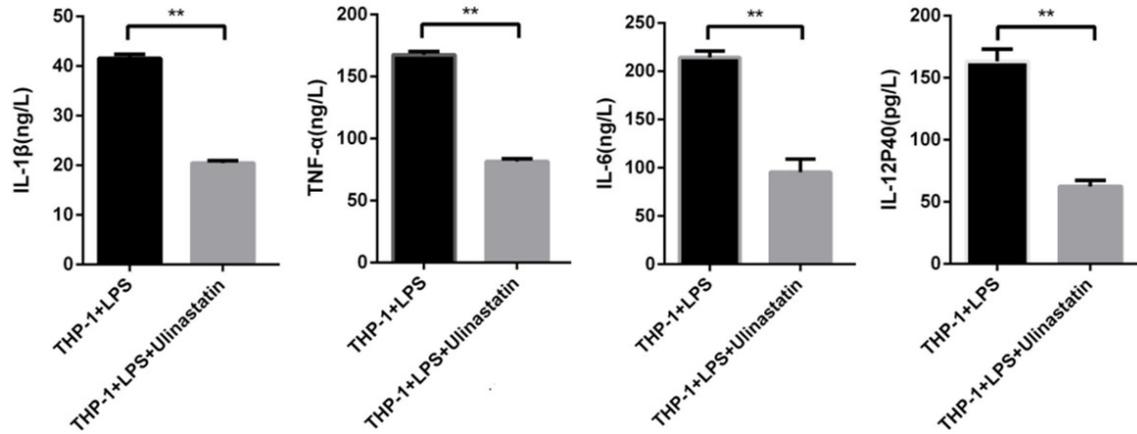


Figure 1. Effects of ulinastatin on the secretion of proinflammatory cytokines by LPS-induced THP-1 macrophages. Ulinastatin (2000 U/mL) was added to LPS-induced THP1 macrophages, and then concentrations of IL-1 β , TNF- α , IL-6, and IL-12 were tested by ELISA. ** $P < 0.01$.

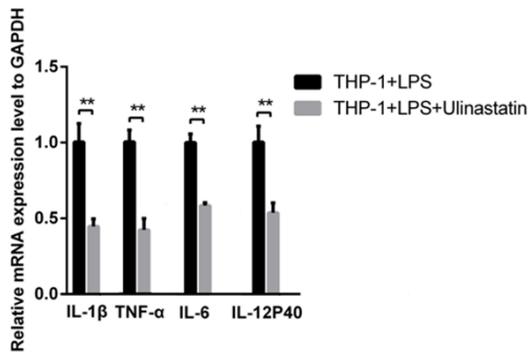


Figure 2. Effects of ulinastatin on the secretion of proinflammatory cytokines in LPS-induced THP-1 macrophages. Ulinastatin (2000 U/mL) was added to LPS-induced THP1 macrophages, and then mRNA of IL-1 β , TNF- α , IL-6, and IL-12 were tested by real-time PCR. ** $P < 0.01$.

Statistical analysis

The data were analyzed for statistical significance using the GraphPad Prism software. Data were presented as mean \pm standard deviation (SD). Student unpaired t test and analysis of variance with Bonferroni post hoc test were used to determine significance. A P value < 0.05 was considered to be statistically significant.

Results

Ulinastatin reduced the secretion of proinflammatory cytokines by LPS-induced THP-1 macrophages

THP-1 cells differentiated into macrophage-like cells after PMA (200 nM) treatment of THP1

cells for 48 h. LPS (100 μ g/mL) were used to treat THP-1 macrophages for 24 h. Ulinastatin (2000 U/mL) was added to LPS-induced THP1 macrophages to investigate the inhibitory effect of ulinastatin on the release of proinflammatory factors. ELISA was used to detect the secretion of proinflammatory cytokines. Ulinastatin could significantly reduce the secretion of IL-1 β ($P < 0.01$), TNF- α ($P < 0.01$), IL-6 ($P < 0.01$), and IL-12 ($P < 0.01$) by macrophages (**Figure 1**).

Ulinastatin reduced the mRNA expression of proinflammatory cytokines in LPS-induced THP-1 macrophages

The mRNA expression of proinflammatory cytokines in macrophages after treatment with ulinastatin was examined to verify the effects of ulinastatin on inflammatory cytokines via regulating the gene expression of proinflammatory cytokines. Ulinastatin reduced mRNA expression of IL-1 β ($P < 0.01$), TNF- α ($P < 0.01$), IL-6 ($P < 0.01$), and IL-12 ($P < 0.01$) in THP-1 macrophages (**Figure 2**).

Ulinastatin inhibited the expression of HLA-DR and upregulated CD163 in THP1 macrophages

The expression of HLA-DR and CD163 in macrophages was the marker of M1 and M2. Therefore, the expression of HLA-DR and CD-163 in ulinastatin-treated THP1 macrophages was detected by flow cytometry. As shown in **Figure 3**, ulinastatin treatment decreased the expression of HLA-DR in LPS-induced THP1 macrophages ($P < 0.01$) and increased the expressions of CD163 slightly ($P < 0.01$).

Regulatory effects of ulinastatin on macrophages

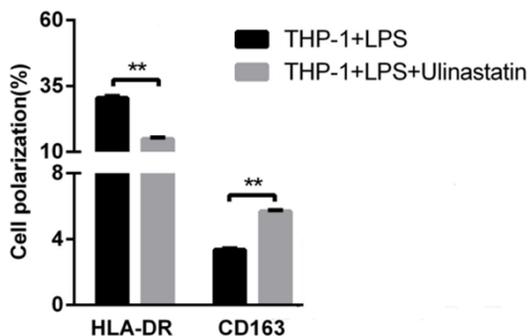


Figure 3. Effects of ulinastatin on HLA-DR and CD163 in LPS-induced THP-1 macrophages. Ulinastatin (2000 U/mL) was added to LPS-induced THP1 macrophages. HLA-DR and CD163 were tested by flow cytometry. ** $P < 0.01$.

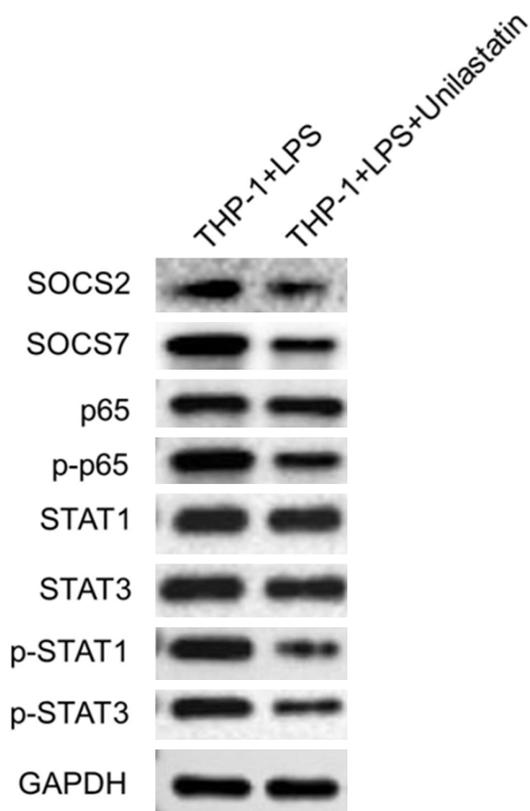


Figure 4. Effects of ulinastatin on total and phosphorylated p65, STAT1, and STAT3 in LPS-induced THP-1 macrophages. Ulinastatin (2000 U/mL) was added to LPS-induced THP1 macrophages. The levels of p65, p-p65, STAT1, p-STAT1, STAT3, and p-STAT3 were tested by Western blotting.

Ulinastatin reduced the phosphorylation of p65, STAT-1 and STAT3 in LPS-induced THP-1 macrophages

The changes in p65 in THP-1 macrophages were not obvious after ulinastatin treatment,

but p-p65 decreased obviously (**Figure 4**). Similarly, STAT1 and STAT3 almost did not change, but p-STAT1 and p-STAT3 decreased (**Figure 4**). The results showed that ulinastatin significantly reduced the inflammation-related signaling pathways.

Discussion

In the present study, ulinastatin directly regulated the mRNA expression of proinflammatory cytokines and secretion by LPS-induced THP-1 macrophages. It also upregulated HLA-DR but downregulated CD163. The phosphorylation of p65, STAT1, and STAT3 in THP-1 macrophages decreased after ulinastatin treatment. The data indicated that ulinastatin could inhibit LPS-induced M1-like characteristics, further confirming its involvement in a variety of inflammation-related diseases.

The polarization of macrophages is vital in the development of sepsis and other diseases. Ulinastatin exerted its protective effects against sepsis and other diseases partly via the regulation of macrophage M1 polarization and reduction of inflammation. Ulinastatin could also interact with other cells, for example, granulocytes, and reduce inflammation [13, 14]. Thus, the therapeutic effects of ulinastatin on diseases such as sepsis are mediated possibly via various types of cells.

Addition of ulinastatin inhibited M1-like characteristics, but also promote M2 polarization. However, the results showed that the proportion of M2 only slightly increased, while the proportion of M1 decreased significantly, indicating that ulinastatin did not polarize all M1 into M2. Too much M2 may cause immune paralysis [15]. Therefore, the effects of ulinastatin on M2 polarization are valuable.

Ulinastatin decreased the phosphorylation of STAT1, STAT3, and p65 in LPS-induced THP1 macrophages, while total STAT1, STAT3, and p65 were not affected obviously. The phosphorylation of STAT1, STAT3, and p65 is an important marker of type M1 macrophages, and it is necessary for M1-type macrophages to secrete inflammatory factors to induce immune responses [16-18]. Ulinastatin regulates the inflammatory response via regulating the phosphorylation of STAT1, STAT3, and p65. However, how ulinastatin regulates this phosphorylation is still not known.

Regulatory effects of ulinastatin on macrophages

In conclusion, ulinastatin regulated THP1 macrophage M1-like characteristics after LPS treatment, suggesting that the therapeutic effect of ulinastatin on various diseases might be due to the regulation of macrophage polarization. The present study provided further evidence for the application of ulinastatin in macrophage-related diseases such as sepsis.

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Disclosure of conflict of interest

None.

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Regulatory effects of ulinastatin on macrophages

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