

Sitafloxacin Inhibits TNF α Release from Monocytic THP-1 Cells Stimulated by LPS

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Sitafloxacin inhibits TNF α release from monocytic THP-1 cells stimulated by LPS

Running head: Sitafloxacin inhibits TNF α release from LPS-stimulated THP-1

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List of Abbreviations

CPFX: ciprofloxacin

ESBL: Extended-spectrum beta-lactamase

ERK: extracellular signal-regulated kinase

GRNX: garenoxacin

IL-8: interleukin-8

IP-10: interferon inducible protein

LPS: lipopolysaccharide

LVFX: levofloxacin

MCP-1: monocyte chemoattractant protein-1

MIP-1 α : macrophage inflammatory protein-1 α

MIP-1 β : macrophage inflammatory protein-1 β

MFLX: moxifloxacin

NF- κ B: Nuclear factor-kappa B

RIPA: radioimmunoprecipitation assay

SIRS: systemic inflammatory response syndrome

STFX: sitafloxacin

TNF- α : tumor necrosis factor- α

Abstract

Sepsis is a systemic reaction to infection and excessive production of inflammatory cytokines and chemokines. It sometimes results in septic shock. The present study was designated to find out which quinolone antibiotic reduces TNF α production the most and to elucidate its mechanisms. We examined which quinolone antibiotic reduced TNF α production from THP-1 cells stimulated by lipopolysaccharide (LPS). Then, we examined the mechanism of inhibition of TNF α production by the antibiotic. STFX most effectively reduced TNF α concentrations within LPS-stimulated THP-1 cells supernatant. STFX suppressed TNF α production in a dose-dependent manner. We found that STFX did not inhibit the NF- κ B, ERK, or p38 pathways, nor did it inhibit the production of TNF α mRNA. The percentage of intracellular TNF α was increased in cells stimulated by LPS and with STFX compared to that of cells stimulated by LPS alone. In conclusion, one of the mechanisms reducing TNF α production from LPS-stimulated THP-1 cells treated with STFX involves inhibition of TNF α release from these cells. STFX has a broad antimicrobial spectrum for gram-positive, gram-negative, and anaerobic bacteria, and may be effective for treating sepsis by both killing bacteria and suppressing inflammation.

Keywords

Sitafloxacin; TNF α , inflammatory cytokines, sepsis; systemic inflammatory response syndrome

Introduction

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. The first definition of sepsis was that of sepsis-1 in 1991, and in 2016 its definition was redefined as sepsis-3. Sepsis was further defined as the systemic inflammatory response syndrome caused by an infection [2]. Particularly during gram-negative bacterial infection, lipopolysaccharide (LPS) stimulates cells to produce inflammatory cytokines and chemokines which can sometimes result in septic shock. Inflammatory cytokines cause decreased blood pressure via dilation of blood vessels and blood clotting within the capillaries of organs. These effects can aid the immune system in the process of fighting infection, but can also be harmful. Thus, drugs that not only fight bacteria but also reduce inflammatory cytokines are required to avoid such harmful effects.

Treatment with such drugs may help to prevent septic shock and reduce mortality. Some antibiotics have been shown to reduce the production of inflammatory cytokines, such as: tetracycline [3, 4], macrolide [5-7], and oxazolidinone [8, 9].

Quinolones, such as garenoxacin or moxifloxacin, have also been reported to reduce inflammatory cytokines [10, 11]. But there remain few reports as to whether sitafloxacin (STFX), a quinolone antimicrobial agent, can lead to reduction in these

cytokines.

STFX exhibits a broad antimicrobial spectrum for gram-positive, gram-negative, and anaerobic bacteria [12]. STFX is an effective agent against pneumococcal infections and incidence of drug-resistant mutants is low in vitro [13]. STFX was shown to be effective against *Haemophilus influenzae* pneumonia in a murine model [14].

In a clinical study, STFX was also proven effective and safe for the treatment of nursing and healthcare-associated pneumonia, including aspiration pneumonia, in elderly patients [15]. STFX treatment was effective for patients with both acute complicated urinary tract infection and pyelonephritis caused by *Escherichia coli* producing extended spectrum beta-lactamase (ESBL) and producing non-ESBL [16]. In another report, STFX was effective against the *E. coli* producing ESBL following 3 days of carbapenem therapy [17].

STFX, a broad-spectrum oral fluoroquinolone, has been approved in Japan for the treatment of respiratory and urinary tract infections. However, it is unknown whether STFX can be used for treating patients with sepsis, or whether it suppresses the production of inflammatory cytokines and chemokines, which we aimed to determine in the current study.

Results

STFX inhibited TNF α production significantly compared with other quinolones

We examined which quinolones exhibited inhibition upon TNF α production by determining the concentration of TNF α within the supernatant of LPS-stimulated THP-1 cells treated with 50 μ g/ml quinolone antibiotics. TNF α concentrations within these supernatants following exposure to LVFX, GRNX, MFLX, CPFX or STFX were 1036.00 \pm 114.65 (pg/mL), 1032.73 \pm 26.42 (pg/mL), 978.05 \pm 45.20 (pg/mL), 792.00 \pm 69.09 (pg/mL), or 286.33 \pm 29.50, respectively. LVFX treatment with LPS stimulation did not significantly reduce TNF α concentration compared with that of LPS alone (1181.20 \pm 20.75 (pg/mL)). However, GRNX, MFXN, CPFX, and STFX treatments significantly reduced TNF α concentrations compared with LPS-only controls ($p < 0.01$). TNF α concentrations following LPS and STFX exposure were significantly lower than those of LPS plus LVFX, GRNX, MFLX, or CPFX ($p < 0.01$), with STFX reducing TNF α concentration the most (Fig. 1).

STFX inhibited TNF α production in a dose-dependent manner

Concentrations of TNF α in the supernatant of LPS-stimulated THP-1 cells in the presence of 10, 30, or 50 μ g/mL STFX were 1766.89 \pm 164.78 pg/mL ($p < 0.05$), 744.87

± 60.72 pg/mL ($p < 0.01$) and 108.91 ± 9.88 pg/mL ($p < 0.01$), respectively. These values were significantly reduced compared to those of THP-1 cells only stimulated with LPS and without STFX treatment (2158.69 ± 24.03 pg/mL) (Fig. 2).

STFX inhibited the production of chemokines

STFX inhibited not only TNF α production but also that of chemokines, as shown by additional experiments with LPS-stimulated THP-1 cells. Concentration of IL-8 in supernatants of cells treated with 50 μ g/ml STFX was significantly decreased to 10472.00 ± 474.67 pg/mL compared with that of LPS alone 17802.33 ± 190.07 ($p < 0.01$) (Fig. 3a). Concentrations of IP-10 in supernatants of cells exposed to 50 μ g/ml STFX was significantly decreased to 77.83 ± 9.70 pg/mL compared with that of LPS alone 3649.00 ± 377.59 ($p < 0.01$) (Fig. 3b). The concentration of MCP-1 in cell supernatants in the presence of 50 μ g/ml STFX was also significantly decreased, to 161.67 ± 11.59 pg/mL compared with that of LPS alone 3453.00 ± 148.55 ($p < 0.01$) (Fig. 3c). Furthermore, MIP-1 α concentrations in the supernatants of cells followed by treatment with 50 μ g/ml STFX were significantly decreased to 9336.67 ± 206.50 pg/mL compared with that of LPS alone 20859.33 ± 196.41 ($p < 0.01$) (Fig. 3d). The supernatant concentration of MIP-1 β from cells exposed to 50 μ g/ml STFX were

additionally significantly decreased to 2844.67 ± 135.43 pg/mL compared with that of LPS alone 12950.67 ± 409.62 ($p < 0.01$) (Fig. 3e).

STFX did not inhibit the signaling pathway

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without the presence of STFX (50 μ g/mL) for 1 hr. The phosphorylated forms of NF-kB, ERK and p38 did not decrease within the cells treated with STFX plus LPS compared to LPS alone (Fig. 4).

STFX did not influence TNF α mRNA levels

Expression of TNF α mRNA was measured in THP-1 cells stimulated with LPS (0.1 μ g/mL) for 1 or 2 hr with or without STFX (50 μ g/mL). TNF α mRNA levels within cells treated with STFX did not significantly change compared with those of LPS alone after either 1 or 2 hrs of exposure (Fig. 5).

STFX inhibit TNF α release from cells

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL). After 4 hr of incubation, intracellular TNF α was stained by anti-TNF α

antibody PE. The percentage of intracellular TNF α in cells treated with STFX and LPS was increased to 16.2% from 4.4% within cells treated with LPS alone (Fig. 6).

Discussion

TNF α plays a very important role within sepsis. Blocking of TNF α has been shown to protect mice from the symptoms of sepsis [18]. Some clinical studies investigating the monoclonal antibodies produced against TNF α for patients with sepsis or septic shock have been reported [19-21]. Modulation of TNF α and other inflammatory cytokines and chemokines is considered very important in the treatment of severe infectious diseases, especially that of sepsis or septic shock.

In the present study, STFX significantly reduced the concentration of TNF α in the supernatants of LPS-stimulated THP-1 cells as compared with other quinolone antibiotics; STFX also reduced the levels of some inflammatory chemokines.

Some types of antibiotics can modulate inflammatory cytokines, but the mechanisms of cytokine inhibition may vary. It has already been reported that minocycline (MINO) inhibits I κ B kinase α / β phosphorylation of the NF- κ B pathway in THP-1 cells [4]. It was also reported that clarithromycin (CAM) attenuates STAT6 phosphorylation.⁵ Other groups reported that macrolide antibiotics decreased functions of the ERK and NF- κ B signaling pathways [6, 7]. GRNX and MFLX were reported to inhibit these signaling pathways to suppress the production of inflammatory cytokines. GRNX significantly inhibited the transcription and secretion of IL-8 induced by

LPS-stimulated THP-1 cells through inhibitory ERK1/2 phosphorylation [10]. Furthermore, MFLX inhibited ERK1/2, JNK, and NF- κ B activation in a cystic fibrosis epithelial cell line [11].

Even when using similar quinolone antibacterial drugs, the mechanism of cytokine suppression differs depending upon the characteristics each drug. Previous studies reported that quinolones with a cyclopropyl group at the N1 position and/or a piperazinyl group at the C7 position, can modify inflammatory responses [22-24]. STFX consists of a fluorocyclopropene at the 1-position of the quinolone skeleton, a chlorine group at the 8-position, a spiroheptane group at the 7-position, and a quinolone with a chlorine group introduced at the 8-position. Such characteristics are considered to cause differences within the spectrum of antibacterial activity, but may also cause differences in anti-inflammatory effects.

In the present study, STFX suppressed TNF α production more strongly than the other tested quinolone antibiotics. It did not suppress the signaling pathways that produced TNF α , nor did it suppress TNF α mRNA production. It is possible that STFX may inhibit the process of producing TNF α from mRNA, or may inhibit the release of produced TNF α from THP-1 cells. Flow cytometry analysis suggested that STFX inhibited the extracellular release of TNF α , but this tendency is slight, and it is

unknown whether this mechanism alone suppressed $\text{TNF}\alpha$ production. One of the mechanisms inhibiting $\text{TNF}\alpha$ production by STFX may not be due to inhibition of the signaling pathways but instead interference with $\text{TNF}\alpha$ release from cells. However, a mechanism inhibiting $\text{TNF}\alpha$ production from mRNA may also be involved and warrants further verification.

STFX may be an effective drug for patients with bacterial infection due to its antimicrobial action and simultaneous reduction of $\text{TNF}\alpha$. STFX is approved as an oral antibacterial drug, and could be a candidate used to treat patients exhibiting sepsis or septic shock.

Methods

Reagents

Roswell Park Memorial Institute (RPMI) 1640 medium and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moxifloxacin (MFLX), garenoxacin (GRNX), and ciprofloxacin (CPFX) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Levofloxacin (LVFX) and sitafloxacin was kindly given from Daiichi Sankyo Company, Limited. These antibiotics were diluted with RPMI 1640 at a concentration of 1.0 mg/mL to create stock solutions. LPS from *Pseudomonas aeruginosa* Serotype 10 (Sigma-Aldrich) was used to induce inflammatory responses. LPS was dissolved in RPMI 1640 medium at a concentration of 1.0 mg/mL and stored at -80°C until use.

Cell culture and exposures

The human monocyte THP-1 cell line was purchased from the RIKEN Cell Bank (Ibaragi, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in humidified air with 5% CO_2 , and only exponentially growing cells were used for experiments. THP-1 cells (2×10^5 cells/mL) were cultured with 0.1 $\mu\text{g/mL}$ of LPS in the presence or absence of antibiotics (MFLX, GRNX, LVFX,

CPF α , and STF α) for 4 h. Following incubation, supernatants were collected via centrifugation at 1,500 rpm for 2 min at room temperature and stored at -80°C until further analysis.

TNF α ELISA assay

ELISA was performed with the TNF α Human ELISA Kit (Invitrogen, Carlsbad, CA, USA) for the determination of TNF α concentration. Samples were read with an automated plate reader (Multiskan Spectrum; Thermo Scientific, Waltham MA, USA).

Multiplex bead immunoassays

Multiplex bead immunoassays (Bio-Plex Suspension Array System, BIO-RAD Laboratories, Inc., CA, USA), which incorporate novel technology with color-coded beads and permits the simultaneous detection of up to 100 cytokines and chemokines in a single well of a 96-well microplate in just 3 hr, was used for the simultaneous quantification of the following chemokines: interleukin-8 (IL-8), interferon inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α) and macrophage inflammatory protein-1 β (MIP-1 β).

Western blot analysis

Total protein was extracted from LPS-stimulated cells treated with antibiotics by using 200 μ l radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing a protease inhibitor cocktail (Roche, Basel, Switzerland) and phosphatase inhibitor. The lysates were clarified by centrifugation (15,000 rpm, 10 min, 4°C). The protein concentration was determined using a Protein Assay BCA Kit (Nacali Tesque Inc., Kyoto, Japan). Samples containing 10 μ g of protein were run on 10% polyacrylamide gel and electrotransferred onto polyvinylidene fluoride filter membranes. The membranes were blocked for 60 min at room temperature in 1% bovine serum albumin in Tris-buffered saline containing Tween 20, followed by overnight incubation with the primary antibody, anti-phospho-NF- κ B p65 rabbit monoclonal antibody (1:1,000; Cell Signaling, Danvers, MA, USA), anti-phospho-ERK rabbit monoclonal antibody (1:1,000; Cell Signaling), or anti-phospho-p38 alpha mouse monoclonal antibody (1:1,000; Cell Signaling) at 4°C. Primary antibody was detected via incubation with horseradish peroxidase-conjugated secondary antibodies and visualized using ImmunoStar Zeta (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Expression of each protein was analyzed by a

Multi Imager II Chemi BOX (BioTools, Gunma, Japan).

RNA extraction and quantification

Total RNA was extracted using Sepasol-RNA I Super G (Nacalai Tesque), cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan), and quantitative PCR analyses were performed using PowerUp™ SYBR Green Master Mix (Thermo Fisher Scientific), according to the manufacturers' protocols. Values were normalized to the housekeeping gene GAPDH on the MX3000P

Real-Time PCR system according to the manufacturer's protocol (Agilent Technologies Inc., Santa Clara, CA, USA). The sequences of specific primer pairs for target genes are available upon request from the website of Takara Bio Inc.

Flow cytometry analysis of intracellular TNF α staining

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL) for 4h. After incubation, cells were fixed and permeabilized using a Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Intracellular TNF α was stained using anti-TNF α antibody PE (BD Biosciences) for 1 h. Cells were washed and resuspended

in PBS supplemented with 2% fetal bovine serum and 0.05% NaN₃. Intracellular TNF α was evaluated using a FACS Canto II (BD Biosciences).

Statistical analysis

Values are presented as average \pm SD. Data were analyzed by using Student's t-test with statistical software (Microsoft Excel 2008; Microsoft Corporation, Redmond, WA, USA), in which a p-value of < 0.05 was considered statistically significant.

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Declaration of Interest

The authors declare no conflict of interest.

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Contributors

I.S., M.F., Y.Y. and H.I. contributed to study design. I.S., M.F. and H.I. contributed to data collection and writing the report. I.S., M.F., K.I., W.O. and K.T. performed experiments. All authors reviewed and approved the final version of the report.

Figure legends

Fig. 1 Sitafloxacin significantly reduced TNF α production

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with several different quinolone antibiotics (50 μ g/mL) for 4 hr. Data shown are the average \pm SD of 3 independent experiments. *p < 0.01 vs LPS alone. **p < 0.01 vs LVFX, GRNX, MFLX, or CPFX. LPS: lipopolysaccharide, LVFX: levofloxacin, GRNX: garenoxacin, MFLX: moxifloxacin, CPFX: ciprofloxacin, STFX: sitafloxacin.

Fig. 2 Sitafloxacin reduced TNF α in a dose-dependent manner

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) in the presence of various concentrations of STFX (1, 10, 30, or 50 μ g/mL) for 4 hr. Data shown are the average \pm SD of 3 independent experiments. *p < 0.05, **p < 0.01 vs LPS alone.

STFX: sitafloxacin

Fig. 3 STFX reduced the levels of inflammatory chemokines

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with STFX (50 μ g/mL) for 4 hr. Concentrations of IL-8 (3a), IP-10 (3b), MCP-1 (3c), MIP-1 α (3d) and MIP-1 β (3e) were measured via multiplex bead immunoassays. Data shown are the

average \pm SD of 3 independent experiments. * $p < 0.01$ vs LPS alone.

IL-8: interleukin-8, IP-10: interferon inducible protein, MCP-1: monocyte

chemoattractant protein-1, MIP-1 α : macrophage inflammatory protein-1 α , MIP-1 β :

macrophage inflammatory protein-1 β .

Fig. 4 STFX did not inhibit signaling of TNF α production

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL) for 15min or 60 min. The phosphorylation of NF- κ B, ERK and p38 was evaluated by western blot analysis. Data are representative of 3 independent experiments.

NF- κ B: Nuclear factor-kappa B, ERK: extracellular signal-regulated kinase.

Fig. 5 Levels of TNF α mRNA were equivalent between cells treated with LPS alone or LPS plus STFX

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL) for 1 hr or 2 hr. Data shown are the average \pm SD of 3 independent experiments.

Fig. 6 Intracellular TNF α levels increased with STFX

THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX

(50 $\mu\text{g}/\text{mL}$). After 4 hr incubation, intracellular $\text{TNF}\alpha$ was stained by anti- $\text{TNF}\alpha$ antibody PE. The percentage of intracellular $\text{TNF}\alpha$ within LPS-stimulated cells with or without the presence of STFX was evaluated by flow cytometry. Data shown are representative of 3 independent experiments.

Figures

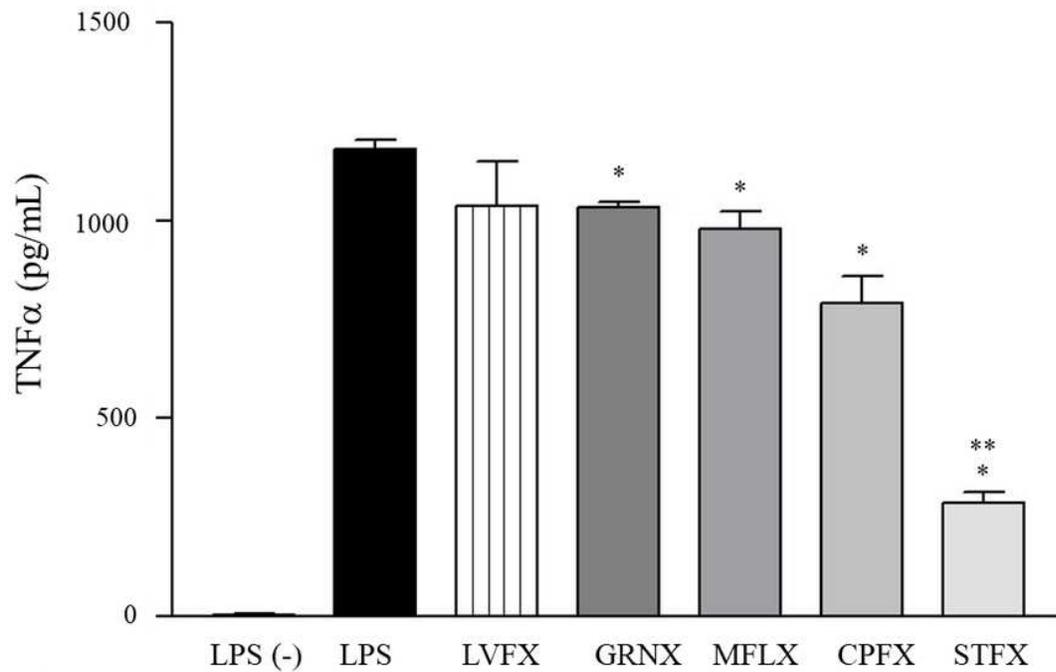


Fig. 1

Figure 1

Sitafloxacin significantly reduced TNF α production THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with several different quinolone antibiotics (50 μ g/mL) for 4 hr. Data shown are the average \pm SD of 3 independent experiments. * $p < 0.01$ vs LPS alone. ** $p < 0.01$ vs LVFX, GRNX, MFLX, or CPFX. LPS: lipopolysaccharide, LVFX: levofloxacin, GRNX: garenoxacin, MFLX: moxifloxacin, CPFX: ciprofloxacin, STFX: sitafloxacin.

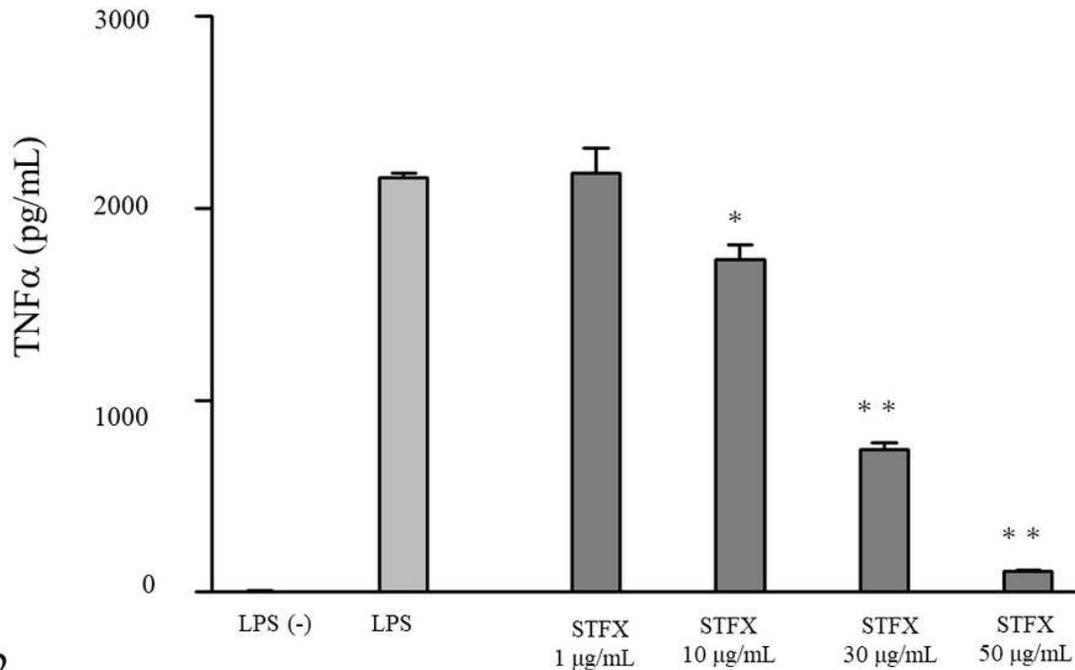


Fig. 2

Figure 2

Sitafloxacin reduced TNFα in a dose-dependent manner THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 µg/mL) in the presence of various concentrations of STFX (1, 10, 30, or 50 µg/mL) for 4 hr. Data shown are the average \pm SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ vs LPS alone. STFX: sitafloxacin

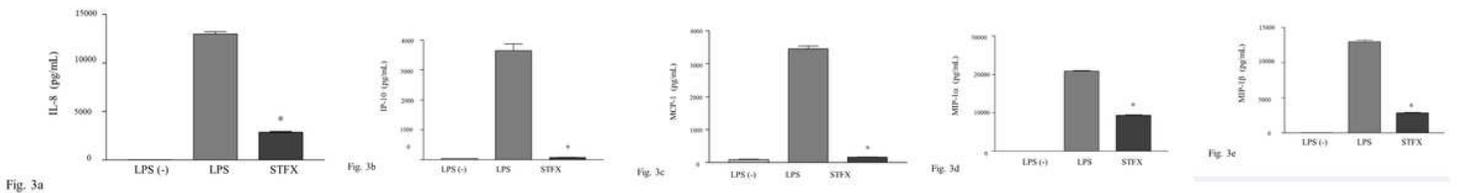


Figure 3

STFX reduced the levels of inflammatory chemokines THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 µg/mL) with STFX (50 µg/mL) for 4 hr. Concentrations of IL-8 (3a), IP-10 (3b), MCP-1 (3c), MIP-1α (3d) and MIP-1β (3e) were measured via multiplex bead immunoassays. Data shown are the average \pm SD of 3 independent experiments. * $p < 0.01$ vs LPS alone. IL-8: interleukin-8, IP-10: interferon inducible protein, MCP-1: monocyte chemoattractant protein-1, MIP-1α: macrophage inflammatory protein-1α, MIP-1β: macrophage inflammatory protein-1β.

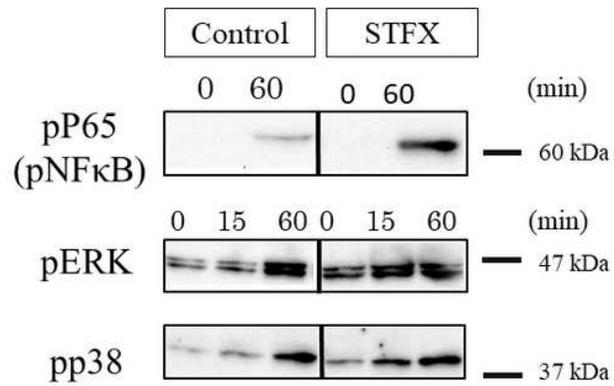


Fig. 4

Figure 4

STFX did not inhibit signaling of TNF α production THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL) for 15min or 60 min. The phosphorylation of NF- κ B, ERK and p38 was evaluated by western blot analysis. Data are representative of 3 independent experiments. NF- κ B: Nuclear factor-kappa B, ERK: extracellular signal-regulated kinase.

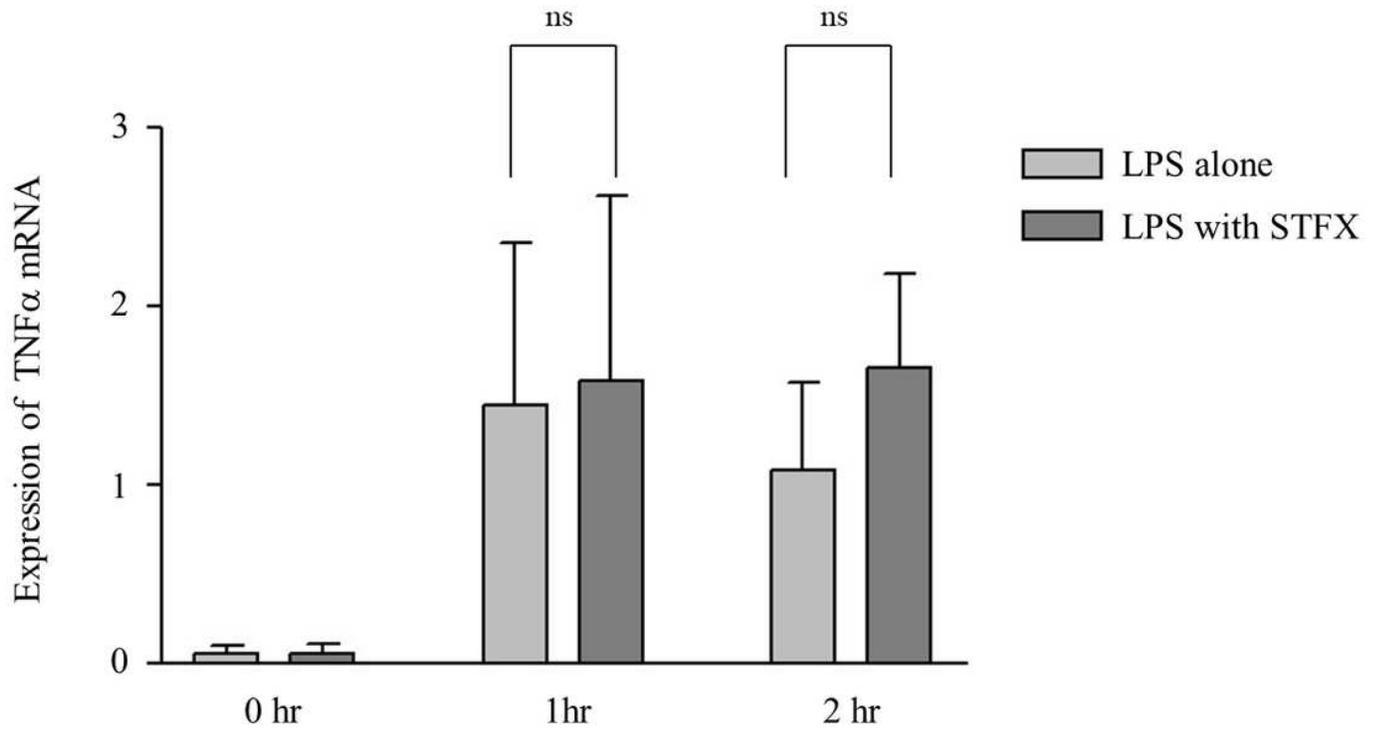


Fig. 5

Figure 5

Levels of TNFα mRNA were equivalent between cells treated with LPS alone or LPS plus STFX THP-1 cells (2×10^5 /mL) were stimulated by LPS ($0.1 \mu\text{g}/\text{mL}$) with or without STFX ($50 \mu\text{g}/\text{mL}$) for 1 hr or 2 hr. Data shown are the average \pm SD of 3 independent experiments.

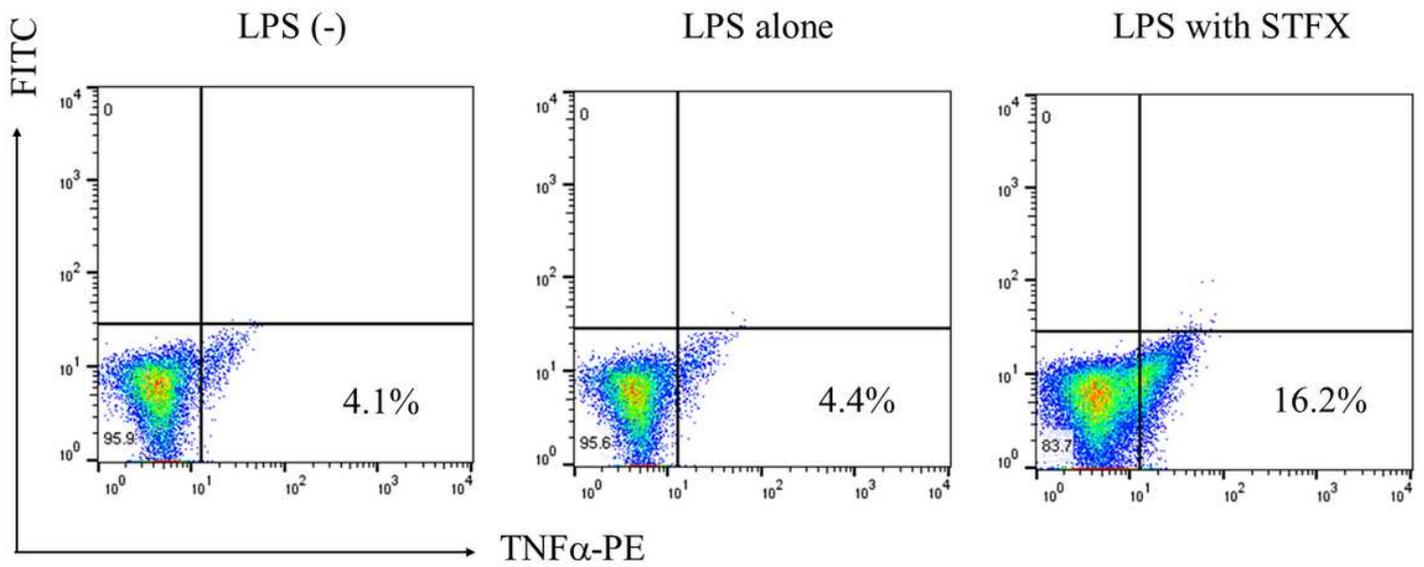


Fig. 6

Figure 6

Intracellular TNF α levels increased with STFX THP-1 cells (2×10^5 /mL) were stimulated by LPS (0.1 μ g/mL) with or without STFX (50 μ g/mL). After 4 hr incubation, intracellular TNF α was stained by anti-TNF α antibody PE. The percentage of intracellular TNF α within LPS-stimulated cells with or without the presence of STFX was evaluated by flow cytometry. Data shown are representative of 3 independent experiments.