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BCL6 inhibitor FX1 attenuates inflammatory responses in murine sepsis through strengthening BCL6 binding affinity to downstream target gene promoters

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ABSTRACT

Background: Sepsis occurs when an infection triggers deranged inflammatory responses. There exists no efficacious treatment for this condition. The transcriptional repressor B-cell Lymphoma 6 (BCL6) is known to act as an inhibitor of macrophage-mediated inflammatory responses. FX1, a novel specific BCL6 BTB inhibitor, is able to attenuate activity of B cell-like diffuse large B cell lymphoma (ABC-DLBCL). Nevertheless, the effect of FX1 in inflammatory responses and sepsis remains unknown.
Objectives: Here, we explored the effect and potential mechanisms of FX1 on the regulation of LPS-induced inflammatory responses in murine sepsis.
Method: Mice models of LPS-induced sepsis were monitored for survival rate following FX1 administration. ELISA was used to assess how FX1 administration affected pro-inflammatory cytokines present in macrophages exposed to LPS and in the serum of mice sepsis models. Flow cytometric analysis, Western blot and qRT-PCR were performed to evaluate differences in macrophages immune responses after FX1 pre-treatment. Finally, the affinity of BCL6 binding to downstream target genes was checked by ChIP.
Results: The survival rate of mice models of LPS-induced sepsis was improved in following FX1 administration. FX1 decreased the production of inflammatory cytokines, attenuated macrophage infiltration activities and reduced monocytes chemotaxis activities, all of which suggest that FX1 exert anti-inflammatory effects. Mechanistically, FX1 may enhance the affinity of BCL6 binding to downstream target pro-inflammatory genes.
Conclusions: These findings illustrated the anti-inflammatory properties and potential mechanisms of FX1 in sepsis caused by LPS. FX1 could potentially become a new immunosuppressive and anti-inflammatory drug candidate in sepsis therapy.

1. Introduction

Sepsis is a life-threatening clinical phenomenon caused by a dysfunctional immune system response to infectious pathogens and may culminate in multi-organ failure [1]. It is the second leading cause of death in the intensive care unit (ICU) setting, carrying with it an extremely high mortality rate [2]. The early phases of sepsis occurs as a consequence of a complex interaction between an overwhelming

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production of pro-inflammatory mediators as well as the activation of several counter-regulatory mechanisms finally leading to multi-organ damage and death [3,4].

Sepsis remains a critical public health issue with no improvement in hospital mortality in the past decade despite vast research investigating the pathophysiological mechanisms of sepsis and advances in sepsis-specific treatment and supportive care [5]. It is imperative that novel mechanistic targets in sepsis be discovered in order to facilitate the development of a clinically efficacious anti-sepsis drug.

Macrophages play a central role in mediating the innate immune response by mounting initial resistance against invading pathogens [6,7]. Toll-like receptors present on the surface of macrophages are responsible for pathogen recognition, triggering the activation of transcription factor nuclear factor B (NF-κB) which then leads to the production of a cascade of proinflammatory cytokines including MCP-1, tumor necrosis factor-α (TNF-α), IL-1β and IL-6, and MCP-1 [8]. NF-κB proteins are important in initiating and subsequently expanding the inflammatory response [9,10]. These molecules remain in an inactivated state in the cytoplasm, bound to inhibitory proteins IκBs. Activated NF-κB consisting of subunits p50 and p65 immediately translocates into the nucleus to initiate pro-inflammatory mediator transcription [11,12]. Therefore, inflammatory disease treatment has been hypothesised to benefit from NF-κB pathway inhibition.

The transcriptional repressor B-cell Lymphoma 6 (BCL6) is primarily present in germinal center B cells (GC-B cells). BCL6 induces GC-B cell survival and proliferation through inhibition of a number of checkpoint pathways by recruiting the BCOR (BCL6 corepressor) co-repressors to its N-terminal BTB domain, NCOR (Nuclear Receptor Corepressor) as well as SMRT (silencing mediator for retinoid or thyroid-hormone receptors) [13–15]. BCL6 is able to regulate the germinal center response by counter-regulating activation of NF-κB. BCL6-induced attenuation of NF-κB signaling has been proposed to take place via a variety of ways including inverse regulation of NF-κB target genes, inhibitory binding of BCL6 to NF-κB proteins and direct p50 inhibition of p50 [16,17]. BCL6 is transiently induced in macrophages and goes on to directly inhibit CCL2 (MCP-1) expression in a NF-κB–dependent manner, suggesting that BCL6 is able to repress acute inflammatory responses [18–20].

FX1 is a novel specific BCL6 BTB inhibitor that is designed to bind to BCL6 at a key region of the its lateral groove and has higher potency than endogenous corepressors. FX1 reactivates BCL6 target genes, disrupts the BCL6 inhibitory complex, and mimics mice phenotypes that have been genetically modified to express BCL6 with corepressor binding site mutations. Minute FX1 doses are able to induce regression of tumors in mice with DLBCL xenografts. In addition, ex vivo primary human ABC-DLBCL samples as well as in vivo and in vitro ABC-DLBCL cells are able to be repressed by FX1 [21]. Therefore, we speculate that FX1 may be involved in the control of macrophage activities. However, it is unclear whether FX1 is involved in the regulation of macrophage-facilitated immune responses induced by LPS. Our goals in the current study are to explore the roles of FX1 in manipulating macrophage activity in vivo and in vitro and to reveal its possible mechanisms. Our results demonstrated that FX1 exerts its anti-inflammatory effects by enhancing the affinity of BCL6 binding to downstream target genes promoters in LPS-stimulated macrophages and LPS-challenged mice.

2. Materials and methods

2.1. Cell culture, drugs and transfection

Mouse monocyte/macrophage RAW264.7 cells were purchased from ATCC (Manassas, USA). Cell lines were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS (FBS; Hyclone, Logan, UT), 100 μg/ml streptomycin and 100 U penicillin. The FuGENE HD transfection reagent (Promega, USA) was used to carry out transfection based on manufacturer's protocols.

Different concentrations of lipopolysaccharide (Sigma, USA) (LPS) were produced by dissolving it in PBS. Similarly, DMSO was used to dissolve FX1 (Selleck, China). Several concentrations of FX1 were used tested to determine a concentration that preserved viability of RAW264.7 cells, and 25 μM FX1 was finally determined to be a suitable concentration for this study.

2.2. Cell viability

96-well plates were used to seed RAW 264.7 cells (1×10^4 cells/well). The Escinor vehicle (normal saline) of varying concentrations was then used to treat the cells for 24 h. The Cell Counting Kit-8 (Beyotime, China) was used to determine RAW264.7 cell viability in strict compliance to manufacturer's protocols. Experiments were performed in triplicate.

2.3. Isolation of BMDMs

Bone Marrow-derived Macrophages (BMDMs) from 7-to-9-week-old male C57BL/6 mice were generated as per previous protocols. Briefly, mouse bone marrow cells were exposed to DMEM supplemented with 20% M-CSF (Peprotech, USA) and 10% FBS for 7 days in order for them to differentiate. Differentiated cells were subsequently subjected to an overnight culture in 96- and 6-well plates at densities of 2×10^4 and 8×10^5 cells/well, respectively.

2.4. Animals and treatment

8–12 week old male C57BL/6 mice were reared and kept in typical facilities used at our center. All animals were handled ethically, with guidelines formulated based on the US National Institutes of Health (NIH) conducted animal care and use guidelines and pre-approved by the Ethics Committee of Huazhong University of Science and Technology Union Hospital. Sepsis was induced by injecting each mouse with 10 mg/kg of intraperitoneal LPS. Mice were exposed to either vehicle (DMSO) or FX1 1 h prior to the LPS (10 mg/kg) challenge. Blood samples were harvested from the inferior vena cava in order to quantify serum cytokines. Three independent experiments were conducted.

2.5. Blood sample and peritoneal fluid preparation

16 h post-LPS challenge, 8 ml of cold PBS was injected intraperitoneally injected and peritoneal fluids drained after 1 min of gentle massage. Intracardiac puncture was used to collect blood at specific durations after LPS treatment and sera were prepared by centrifuging blood for 20 min at 3000 rpm for ELISA analysis.

2.6. ELISA and Western blot

TNF-α, MCP-1, IL-1β, IL6, IL-10 and TGFβ1 concentrations in the serum or culture media were quantified with commercial ELISA kits supplied by the Neobioscience Technology Company.

Cells were harvested at pre-specified times and homogenized in ice-cold RIPA Lysis Buffer (Beyotime, China) that contained with 1 mM protease inhibitor (phenyl methanesulfonyl fluoride, PMSF, Beyotime, China). The enhanced BCA Protein assay kit (Beyotime, China) was used to quantify proteins. Proteins were separated via electrophoresis with a SDS-PAGE gel, electroblotted onto nitrocellulose membranes and incubated at 4 °C overnight with the following antibodies: BCL6 (Abcam, USA), β-actin (ABclonal, China), phospho-p65 and P65 (CST, USA), phospho-p38 and p38 (CST, USA), BCOR (ABclonal, China), phospho-Erk and Erk (CST, USA), phospho-Jnk and Jnk (CST, USA), The following morning, proteins were incubated with peroxidase-conjugated secondary antibody and final images analyzed with a Bio-Rad (Hercules, CA) imaging system.

2.7. Plasmids construction

A pGL6-TA vector containing a pGL6-NF-κB-Luc reporter with four NF-κB response elements (GGGAATTTCC) was obtained from the Beyotime Institute of Biotechnology.

2.8. Luciferase reporter assay

The luciferase reporter construct was co-transfected into RAW264.7 cells with plasmid pRL-TK (renilla luciferase reporter plasmid) that was used as an internal control, and then stimulated with LPS after treatment with FX1 or vehicle (DMSO) for 1 h. Luciferase activity was determined in lysed cells with the Dual Luciferase Reporter Assay Kit (Promega, USA) following the manufacturer's protocols.

2.9. Recombinant adenovirus generation

An adenovirus expression vector kit (Takara Bio, Japan) was used to construct a replication-deficient recombinant adenovirus carrying the entire BCL6 coding sequence (Ad-BCL6). The Ad-control (negative control) was the adenovirus-only green fluorescent protein (GFP). Three siRNAs against mouse BCL6 were designed to generate an adenovirus (Ad-si-BCL6) expressing siRNA against BCL6. The siRNA that had the best knockdown efficiency was selected to be reconstituted into an adenoviral vector. Amplification and purification of the recombinant adenovirus were performed based on the manufacturer's instructions (Takara Bio, Japan).

2.10. Quantitative polymerase chain reaction

Total RNA extraction from tissues and cells were carried out with the TRIzol reagent (Takara Bio, Japan). The RNA PCR Kit (Takara Bio, Japan) was used to reverse transcribe RNA. An ABI PRISM 7900 Sequence Detector system (Applied Biosystem, USA) was used to carry out quantitative polymerase chain reaction (PCR) amplification following the manufacturer's instructions. The comparative Ct M method formula $2^{-\Delta\Delta Ct}$ was used to determine relative gene expressions of TNF-α, MCP-1, IL-1β and IL6, based on endogenous control gene GAPDH. The sequences of the primers used are shown in Table 1.

2.11. Histopathological examinations and histologic injury scores

Hematoxylin and eosin was used to stain liver and lung sections which were then evaluated under light microscopy. Histologic injury was evaluated by a pathologist blind to treatment assignment. Histologic injury scores for the livers, and lungs were assessed according to the following criteria. Liver injury was scored as follows: no damage (0), mild (1), moderate (2), and severe (3) for the following parameters: necrosis, vacuolization, ballooning degeneration, inflammation, erythrocyte stasis, and hepatic cord structure disruption. Lung injury was scored on a scale of 0 to 4 as follows: appears normal (0), mild (1), moderate (2), severe (3), and very severe (4) for infiltration of macrophages into airways or vascular walls, alveolar congestion, hemorrhage, and thickness of alveolar wall.

2.12. Chromatin immunoprecipitation (ChIP)

3×10^7 RAW 264.7 cells were exposed to 25 μM FX1 or vehicle for 60 min before subjected to a 24 hour LPS (1 μg/ml) challenge. 37% formaldehyde (Sigma, USA) was then used to fix cells for 10 min at room temperature. Cross-linking reaction was blocked by the inclusion of 125 mM glycine. Cells were rinsed twice with cold PBS and lysed in lysis buffer (Protease inhibitors, 0.1% w/v SDS, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 5 mM EDTA, 50 mM Tris pH 8, 150 mM NaCl). A Covaris S220 AFA Ultrasonicator (Covaris Inc., Woburn, MA, USA) was used to sonicate cells in order to generate fragments < 400 bp. Sonicated lysates were centrifuged, precleared with ChIP-Grade Protein G Magnetic Beads (CST, USA) and subjected to an overnight incubation at 4 °C with control IgG antibody (CST, USA) or specific BCL6 antibody (BCL6 (D65C10) Rabbit mAb #5650 CST, USA, dilution 1:200). For optimal ChIP results, we used 5 μl of antibody and 10 μg of chromatin (approximately 4×10^6 cells) per immunoprecipitation .30ul ChIP-Grade Protein G Magnetic Beads (CST, USA) were added to the cells at 4 °C for 1 h with gentle rocking to recover the immunocomplexes. RIPA buffer was then used to was these beads twice, increasing the astringency ChIP wash buffers at each step (150 mM NaCl, 250 mM NaCl, 250 mM LiCl), and finally with TE buffer. An elution buffer composed of 100 mM $NaHCO_3$ and 1% SDS was used to elute the immunocomplexes. Cross-linking was reverted by addition of 300 mM NaCl and incubation at 65 °C for at least 5 h. DNA purification was performed with a PCR purification kit (TsingKe, China). A ChIP product fraction served as template in quantitative polymerase chain reaction (PCR) amplification using the ABI PRISM 7900 Sequence Detector system (Applied Biosystem, Foster City, CA) and in 10ul real time PCR reactions using SYBR Green (Vazyme, China). Relative enrichment was assessed with input chromatin standard curves.

The primers of BCL6 target genes promoter were as follows:

CXCR4 F: GACCTGGAGTTTACGGGTGG R: TTCCAACATTGCCGCC TACT
IL6 F: TGCTCATGCTTCTTAGGGCT R: TGCACAATGTGACGTCGTTT
MCP-1 F: AGCCAACTCTCACTGAAGCC R: GGGTGATATGCTGGGA AGGG.

2.13. Statistical analysis

All statistical analyses were performed using SPSS version 19. Data was presented in terms of mean ± S.D. A two-tailed Student's *t*-test enabled analysis for differences between two groups. ANOVA was used to quantify differences between three groups. Overall mice survival were determined with Kaplan-Meier analysis. All values of $P < 0.05$ were marked with an asterisk and were considered to be statistically significant.

3. Results

3.1. FX1 increases the survival rate of mice exposed to LPS and suppresses secretion of proinflammatory cytokines in mouse serum

NF-κB signaling is thought to be necessary for generation of pro-inflammatory cytokines that occurs as part of the innate immune system activation response to sepsis. BCL6 works as a sequence-specific transcriptional repressor and blocks NF-κB signaling by inhibiting BCL6 binding to NF-κB proteins, subsequently deranging NF-κB target gene expressions [16,17]. Theoretically, BCL6 inhibitor FX1 is supposed to exacerbate LPS-induced sepsis. First, to avoid in vivo toxicity of overdose FX1 in mice, a cohort of 10 C57BL/6 mice were treated for 10 days with daily doses of 25 mg/kg/day FX1, after which the animals were sacrificed and analyzed for histological evidence of organ damage (Supplementary Fig. 3). No signs of toxicity, inflammation, or infection were evident from H&E-stained sections of lung, heart, kidney, liver and spleen of the fixed organs from mice treated with FX1 compared with vehicle. To determine FX1 effects on LPS-induced sepsis, we investigated if there was an effect of FX1 on LPS-challenged mice serum concentrations of pro-inflammatory cytokines or on LPS-challenged mice mortality. Vehicle (DMSO) or FX1 (25 mg/kg, i.p.) were administered to the animals for 1 h, which were then injected intraperitoneally with LPS (10 mg/kg). At 18 h post-LPS injection, blood samples were collected for analysis of TNF-α, MCP-1, IL-1β and IL-6 levels. Mice survival was observed 12 hourly for the next 4 days. Beyond our expectations, we discovered that FX1 significantly attenuated MCP-1, IL-1β and IL-6 concentrations in serum after LPS challenge

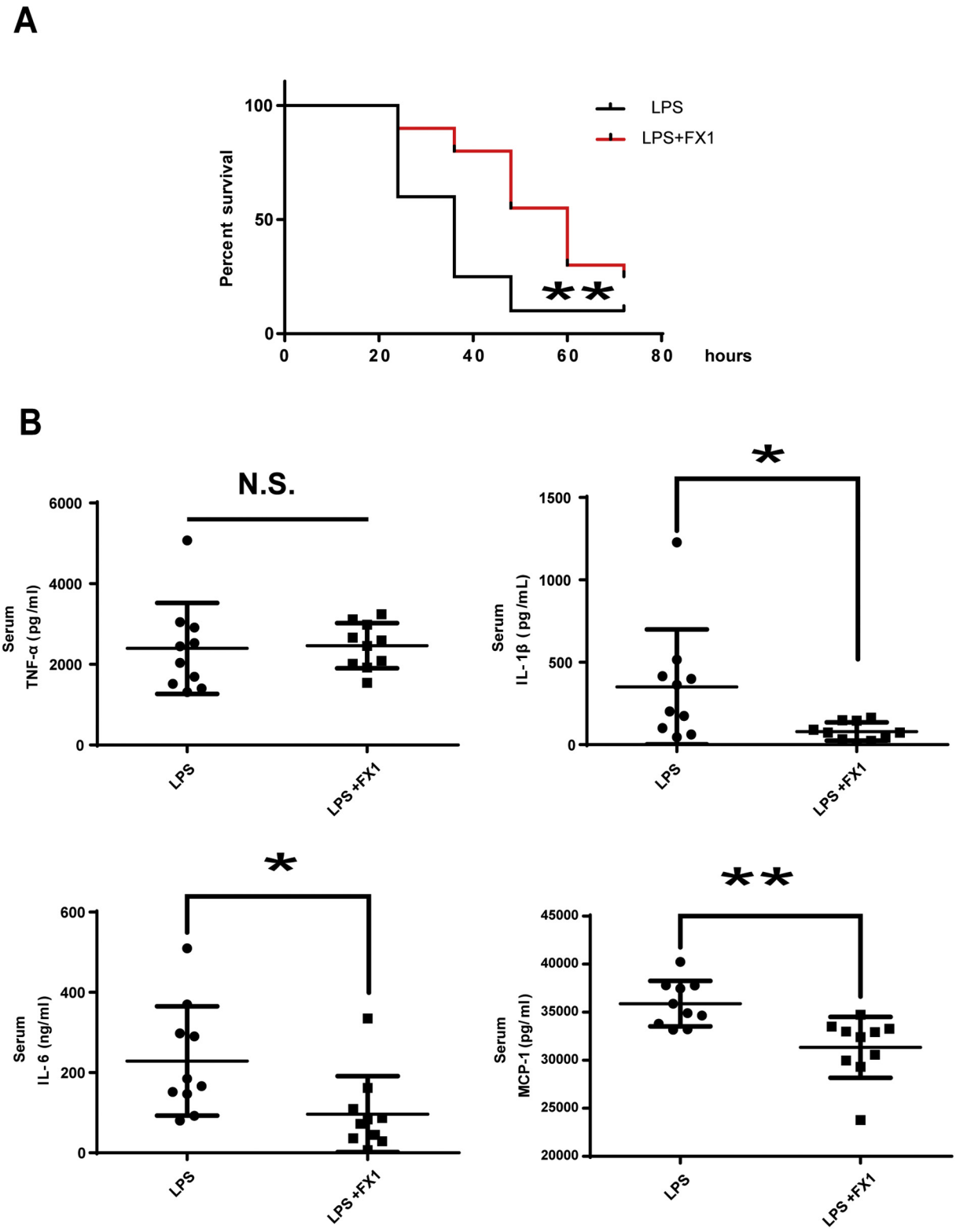
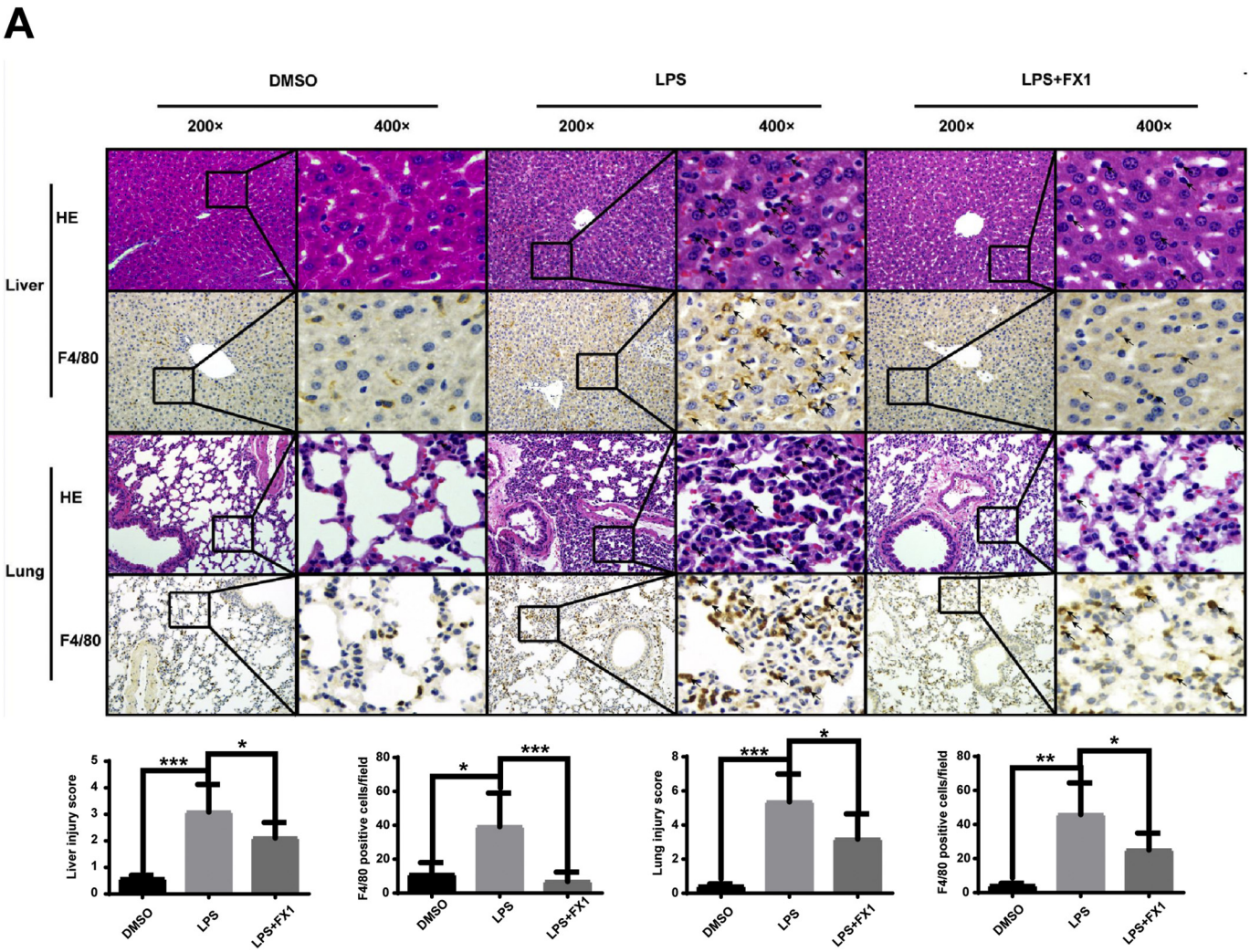


Fig. 1. Effects of FX1 on inflammatory cytokines levels in serum and mortality in LPS-challenged mice.
Male C57BL/6 mice were injected with vehicle (DMSO) or FX1 (25 mg/kg, i.p.) 1 h prior to LPS injection (10 mg/kg, i.p.). Blood samples were collected 18 h after LPS injection. (A) Mice survival were noted 12 hourly for the following 4 days post-LPS injection. Each group had 10 mice. (B) TNF-α, MCP-1, IL-1β and IL-6 levels measured in the mouse serum by ELISA. Statistical significance was evaluating using the Log-Rank test and indicated in the following manner: ˙NS, not significant; *P < 0.05 and **P < 0.01 vs. DMSO group.



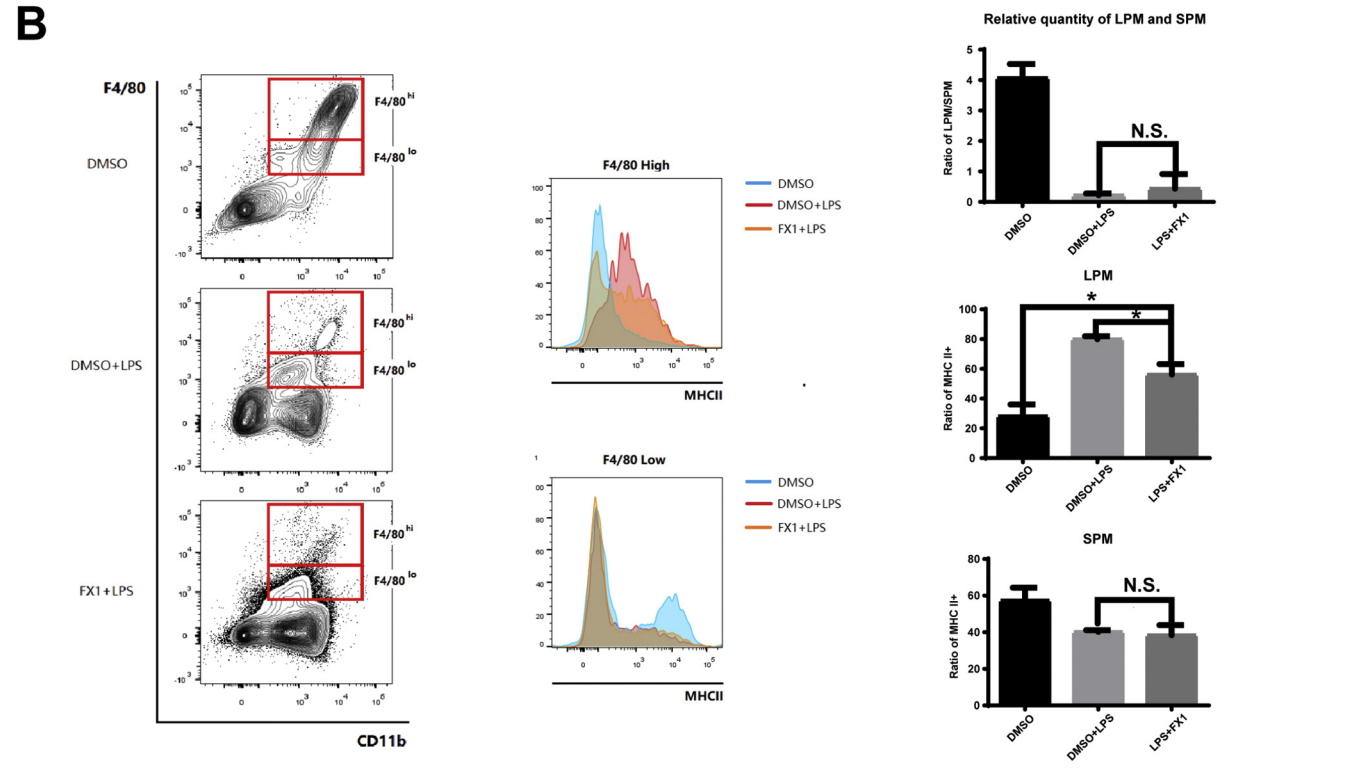


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Fig. 2. FX1 effects on macrophage infiltration and chemotaxis in the lung, liver and peritoneal cavity in vivo after LPS challenge.
C57BL/6 mice were pre-treated with vehicle (DMSO) or FX1 (25 mg/kg, i.p.) 1 h prior to LPS injection (10 mg/kg, i.p.). Liver and lung tissues as well as peritoneal fluids were harvested 18 h after LPS injection. (A) Representative images of H&E-staining and F4/80 immunohistochemical staining of liver tissue sections each group (×200) are shown. Score of liver injury and quantity of 4/80 positive cells was assessed. Representative images of H&E-staining and F4/80 immunohistochemical staining of liver tissue sections from the indicated group (×200) was showed. Score of lung injury and quantity of F4/80 positive cells were assessed. (B) Flow cytometry was used to assess macrophages in peritoneal fluids was quantified with flow cytometry. Representative plots show gated $CD11b^+F4/80^{lo}$ (SPM) and $CD11b^+F4/80^{hi}$ (LPM) macrophages. The relative ratio of these 2 populations within the total $F4/80^+$ gate was similar for DMSO and FX1 pre-treated mice after LPS injection (right top). The changes of MHC II expression in these 2 populations was showed (right down). Data is depicted in terms of mean ± SD (n = 5); ˙NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 vs. LPS group. The figure is a representative of three independent experiments.

(Fig. 1B). Serum concentrations of TNF-α were detectable immediately within 0.5 h and gradually stabilized to normal level within 8 h. There was no significant difference within 18 h. Anti-inflammatory cytokines IL-10 and TGF-beta1 were also detected (Supplementary Fig. 2A). FX1 had no obvious effect on level of IL-10 and TGF-beta1 in serum after LPS challenge. In addition, FX1 also improved survival rates compared to mice who were just exposed to LPS alone (Fig. 1A). These results indicated that FX1 repressed LPS-induced sepsis and inflammatory cytokine production.

3.2. FX1 attenuates infiltration and chemotaxis of macrophages in LPS-challenged mice

Given that FX1 effectively reduced the systemic inflammatory response, we assessed the effects of FX1 on chemotaxis and infiltration of monocytes/macrophages following sepsis. After injection of LPS 18 h, liver and lung samples were harvested and subjected to immunohistochemistry and histopathological examinations. In addition, the peritoneal macrophages were extracted and quantified with flow cytometry. LPS administration obviously increased lung and liver macrophages infiltration (Fig. 2A). FX1 pre-treatment inhibited LPS-induced infiltration of macrophages into the cavities of liver tissue. Lung tissues that were exposed to LPS had decreased numbers of pulmonary alveoli and thicker alveoli walls. FX1 pre-treatment increased numbers of alveoli while attenuated alveolar wall thickening.

There are two kinds of macrophages identified in the peritoneal cavity: $CD11b^+F4/80^{lo}$ small peritoneal macrophages (SPM) and $CD11b^+F4/80^{hi}$ large peritoneal macrophages (LPM) [22]. To analyze the effect of FX1 on activation of macrophages in vivo, we quantified the change of markers in the peritoneal macrophages by flow cytometry at 18 h after LPS injection (Fig. 2B). Compared with the DMSO group, the ratio of SPM was markedly increased and the ratio of LPM were obviously decreased in the LPS group, whereas FX1 had no obvious influence on the ratio of LPM/SPM after LPS stimulated. However, MHC class II activation markers were higher after LPS injection in LPM and FX1 could partially reverse the shift. In SPM, FX1 had no effect. Our data indicated that although FX1 does not alter the relative proportions of peritoneal macrophages subpopulations, it decreases LPM activation and the in vivo M1 proinflammatory state.

In conclusion, FX1 has an inhibitory effect on LPS-induced chemotaxis and infiltration of monocytes/macrophages in vivo.

3.3. FX1 suppresses pro-inflammatory cytokine production in LPS-activated macrophages without change cell viability

To overcome inadvertently using toxic FX1 concentrations, we first tested the effect of FX1 on the survival of RAW 264.7 cells. Various FX1 concentrations (0 μM to 50 μ M) were used to treat cells for 24 h. The CCK-8 assay was then used to evaluate cell viability. Results demonstrate no marked differences in cell viability when RAW 264.7 cells were exposed to FX1 concentrations from 10 to 50 μM (Fig. 3A). To elucidate the effect of FX1 on macrophage activation, RAW 264.7 cells were exposed for 1 h to various concentrations of FX1 and subsequently treated with LPS (100 ng/ml) for 24 h. FX1 significantly attenuated TNF-α, IL-1β, IL-6 and MCP-1 mRNA expressions in LPS-activated RAW 264.7 cells in a concentration-dependent manner without altering BCL6 expression (Fig. 3B). We further detected the effect of FX1 on Bone Marrow Derived Macrophage (BMDM) in at concentration of 25 μM. FX1 dramatically downregulated TNFα, IL-1β, IL-6, MCP-1 expressions at both the mRNA and secretory protein levels of BMDM (Fig. 3C and D). However, FX1 had no effect on level of IL-10 and TGF-beta1 in secretory protein levels of BMDM after LPS challenge (Supplementary Fig. 2B).These findings indicated that FX1 significantly represses pro-inflammatory cytokine production stimulated by LPS.

3.4. Anti-inflammatory effects of FX1 is strongly associated with BCL6

It is seemingly contradictory to the predecessors' research that the above experimental findings revealed the powerful anti-inflammatory effects of BCL6 inhibitor FX1 in LPS-activated macrophages. In order to investigate whether BCL6 was the target of the anti-inflammatory effects of FX1 in LPS-activated macrophages, we constructed a BCL6 gene siRNA recombinant adenovirus vector (Adv-BCL6-siRNA) and its efficacy in knocking down the BCL6 protein was detected in RAW 264.7 cells by qPCR and Western blot (Fig. 4A). Subsequently, BCL6-knockdown and control RAW 264.7 cells were stimulated with LPS, and MCP-1, IL-1β and IL-6 mRNA expression were examined by qPCR analysis. The BCL6-knockdown RAW 264.7 cells showed significantly higher MCP-1, IL-1β and IL-6 mRNA expressions in contrast to control group RAW 264.7 cells. The anti-inflammatory effects of FX1 were weakened after BCL6-knockdown (Fig. 4B). Interpreted as a whole, our findings indicate that BCL6 may have a central function in inhibiting the production of pro-inflammatory cytokines, and that the anti-inflammatory effects of FX1 are largely dependent on BCL6.

3.5. FX1 attenuates LPS-activated MAPK and NF-κB signaling pathways and enhances the affinity of BCL6 binding to downstream target genes promoters

Firstly, to determine whether FX1 could regulate the expression of BCL6 and its corepressors, we next performed western blot to examined BCL6 and BCOR in RAW 264.7 exposed to 100 ng/ml LPS with or without 25 μM FX1 pretreatment for time points. The results showed that FX1 have no obvious influence on the expression of BCL6 and its corepressor BCOR (Fig. 5A).

To characterize signaling pathways involving FX1, Western blot analysis was carried out to quantify activation of NF-κB and MAPK pathways. As shown in Fig. 5B, LPS-activated phosphorylation of these kinases were significantly attenuated by pretreatment with 25 μM FX1 in RAW 264.7 cells. Additionally, reporter gene assay demonstrated that there was a significant FX1 mediated suppression of NF-κB luciferase activities in cells exposed to LPS (Fig. 5C).

Although reducing recruitment of BCL6 corepressor proteins SMRT and BCOR disrupted target gene BCL6 functional repression complex formation [23], FX1 increased binding of BCL6 to its target genes in DLBCL cells [21]. On the other hand, many pro-inflammatory chemokines and cytokines such as MCP-1, IL6 and CXCR4 were genes indirectly repressed by BCL6. To assess if FX1 mediates the repression of pro-inflammatory cytokines and chemokines by BCL6 in macrophages, we next performed quantitative ChIP assays in LPS activated RAW264.7 cells exposed to 25 μM FX1 or not for 24 h. BCL6 binding to known and validated BCL6 binding sites in the promoters of MCP-1, IL6 and CXCR4

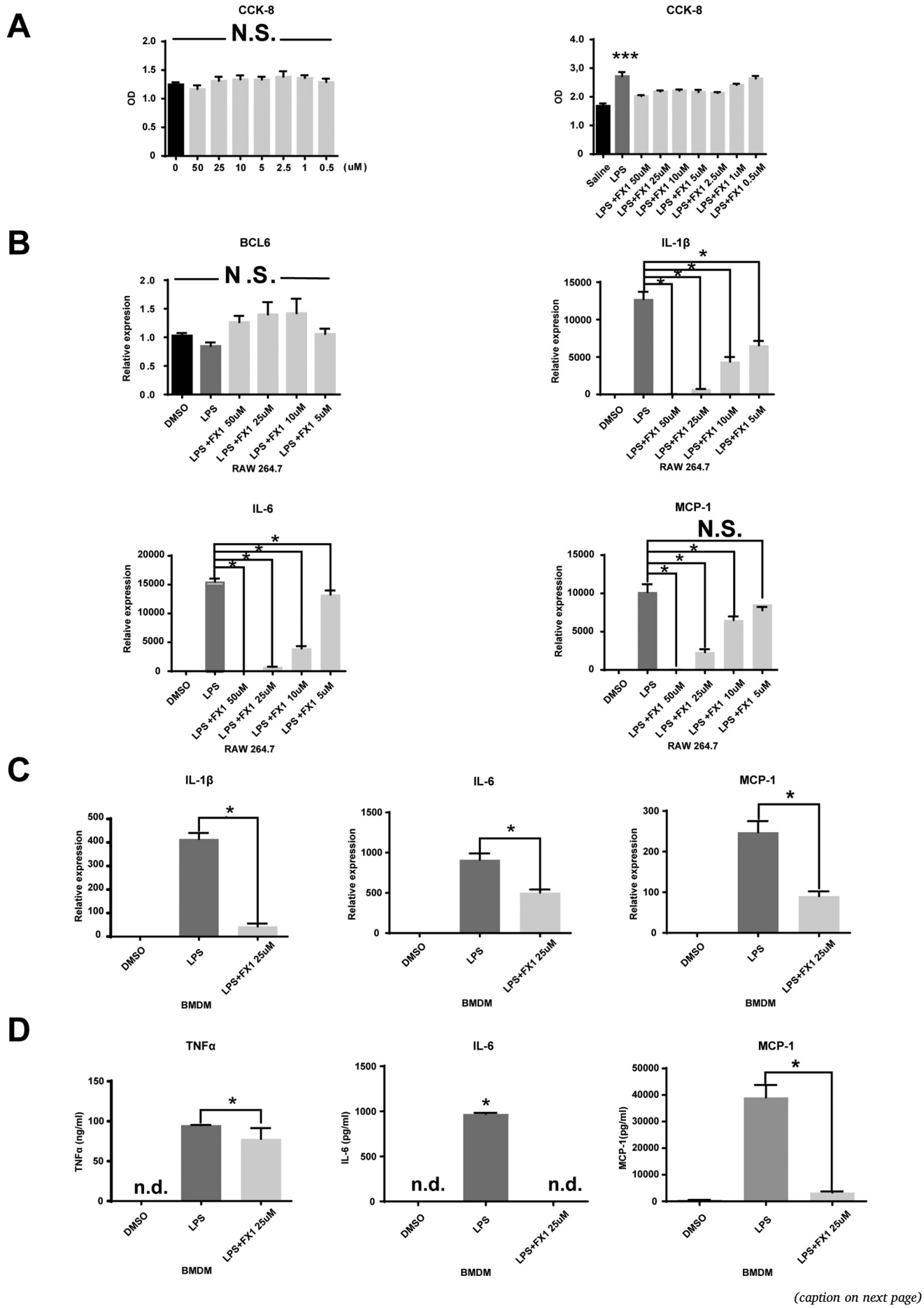

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Fig. 3. Effects of FX1 in LPS-induced macrophage viability, chemokine production and cytokine production in vitro.
(A) RAW264.7 cells were incubated for 1 h with different concentrations of FX1. A CCK-8 assay was used to confirm cell viability. (B) RAW264.7 cells were incubated for 1 h with different concentrations of FX1 and were subsequently exposed to LPS (100 ng/ml) or not for 24 h. mRNA levels of BCL6, IL-1β, IL-6 and MCP-1 of RAW 264.7 cells were measured by qPCR. (C) BMDM were incubated for 1 h with 25uM FX1 concentrations, and then were treated or not treated with LPS (100 ng/ml) for 24 h. mRNA levels of IL-1β, IL-6 and MCP-1 of Bone Marrow Derived Macrophages were measured by qPCR. (D) ELISA was used to measure expressions of TNF-α, IL-6 and MCP-1 in the BMDMs culture supernatant. Data are presented as the mean ± s.d., ˙NS, not significant; *P < 0.05 vs. LPS group.

were examined. After LPS stimulation, binding of BCL6 to promoters of MCP-1, IL6 and CXCR4 were reduced, whereas FX1 apparently reversed this finding (Fig. 5D).

In conclusion, our data indicates that MAPK and NF-κB signaling pathways activation are inhibited by FX1. FX1 also enhances the affinity of BCL6 for binding to downstream target gene promoters, and attenuates macrophage production of pro-inflammatory cytokines.

4. Discussion/conclusion

Recently, Cardenas M. G. et al. identified FX1 to be able to inhibit BCL6 BTB, which reactivated BCL6 target genes and disrupted BCL6 repression complex formation, both of with suppressed in vivo and in vitro growth of DLBCLs cells [21]. In inflammation, although BCL6 has also been shown to function as a regulator of chemokine gene

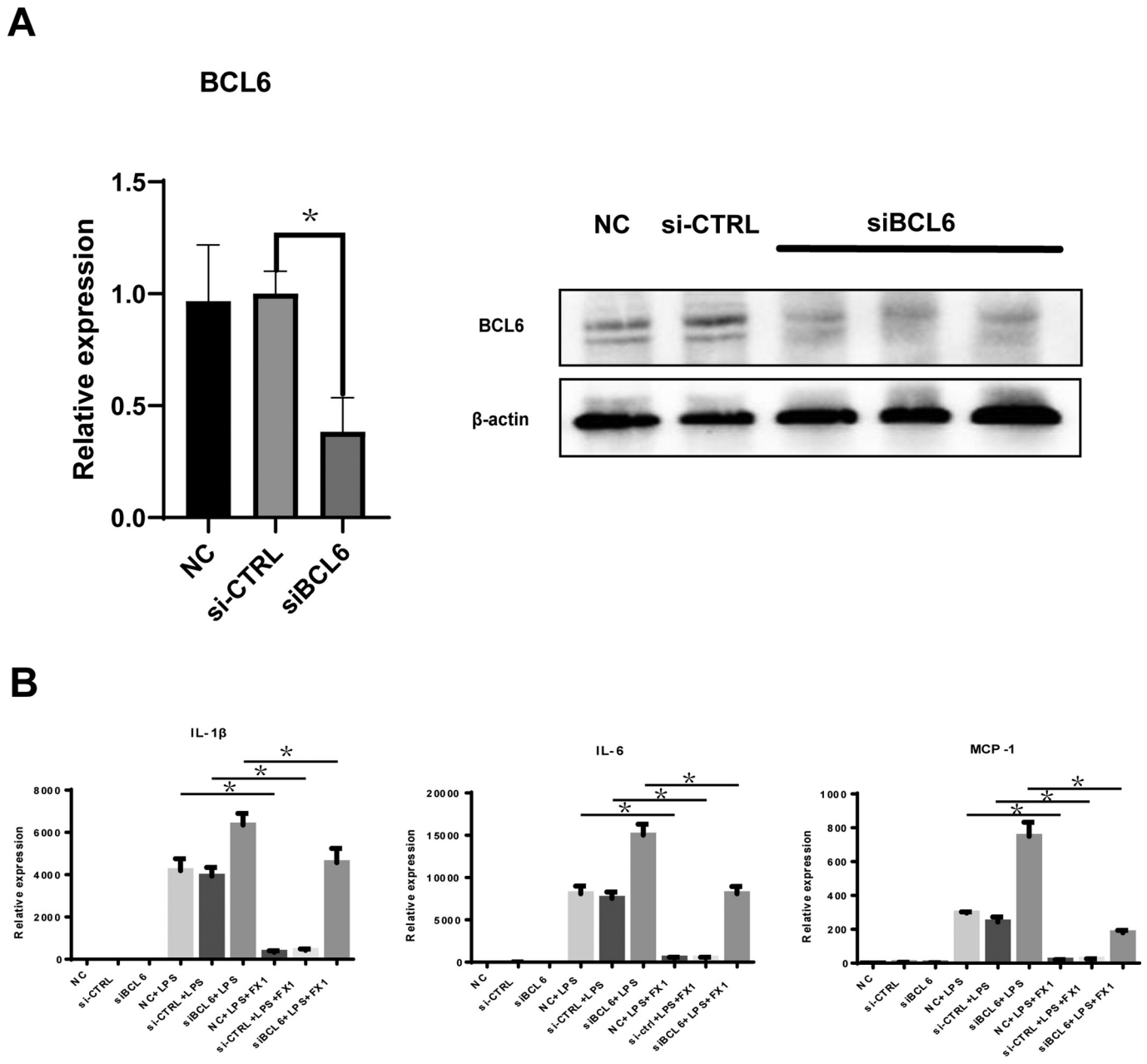
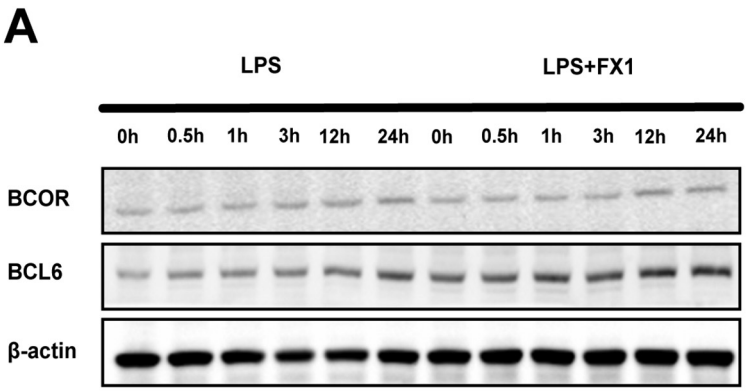
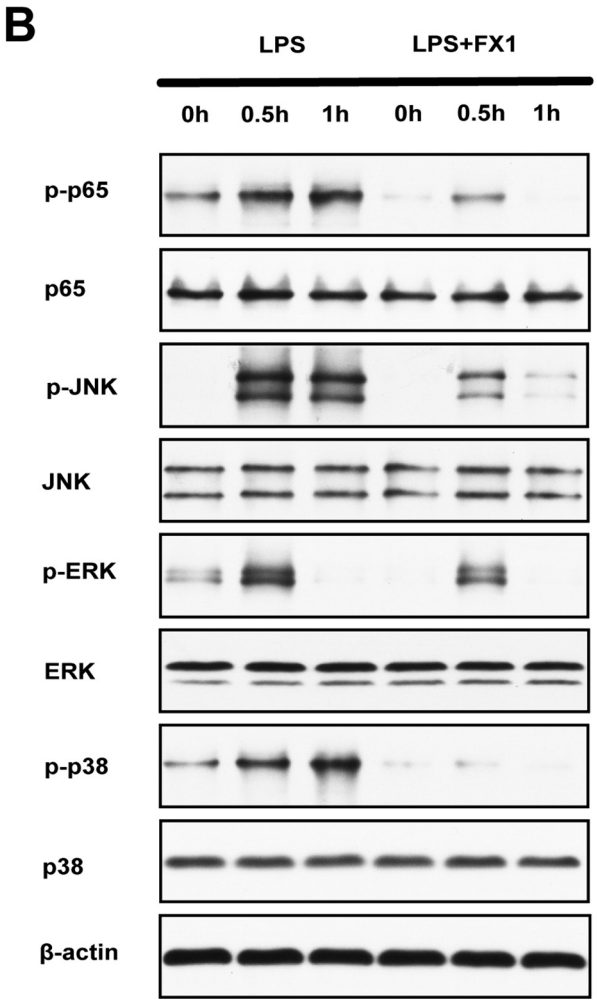
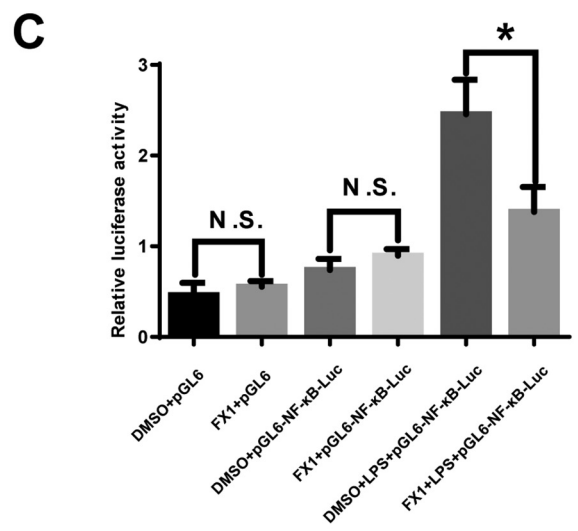


Fig. 4. BCL6 knockdown weakens the anti- inflammatory effect of FX1.
(A) qRT-PCR and western blot were used to assess BCL6 level of knockdown in RAW 264.7 macrophages.
(B) qRT-PCR was used to assess the impact of knocking down BCL6 on inflammatory cytokine expression in RAW 264.7 macrophages exposed to LPS. Data represents the mean ± SEM of three independent experiments. *P < 0.05 vs. NC or si-CTRL group.







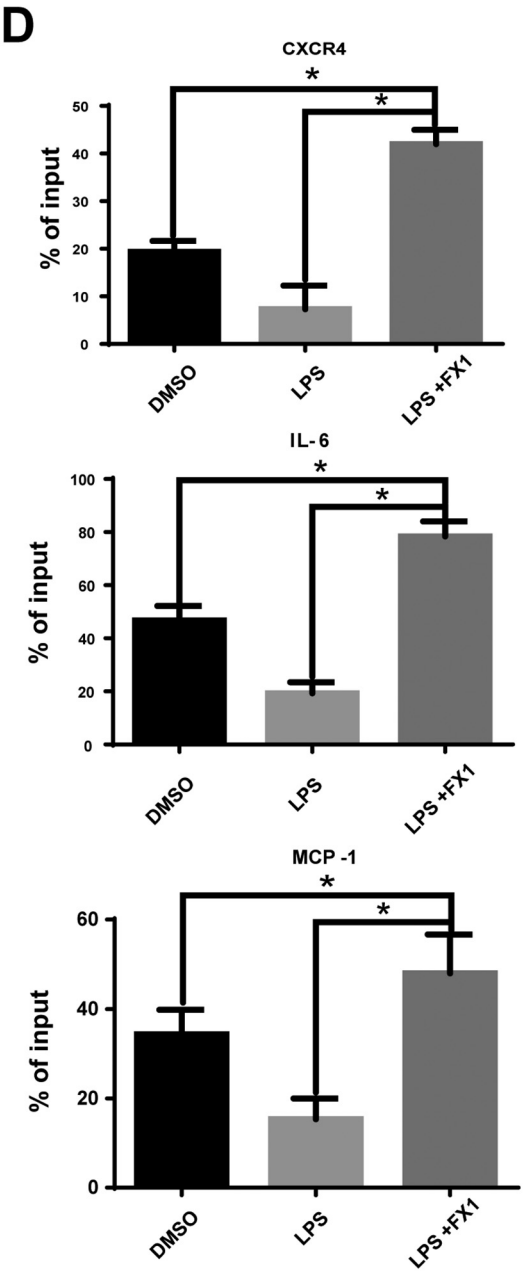


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Fig. 5. FX1 attenuates LPS-activated MAPK and NF-κB signaling pathways and enhances the affinity of BCL6 binding to downstream target genes promoters.
(A) RAW 264.7 macrophages that were either pretreated or not with LPS were used to assess the impact of FX1 on the expression of BCL6 and BCOR, as determined by qPCR at different timepoints. (B) RAW 264.7 macrophages that were either pretreated or not with LPS were used to assess the impact of FX1 on NF-κB and MAPK pathways, as determined by Western blot. (C) Luciferase reporter constructs containing NF-κB promoter was co-transfected with an internal control plasmid pRL-TK into RAW264.7 cells, followed by LPS challenge (100 ng/ml for 24 h). The relative luciferase activities are expressed as a percent of values determined in control group. (D) Quantitative ChIP was performed in RAW 264.7 cells exposed to FX1 or vehicle before LPS stimulated using antibodies for BCL6 or IgG control to enrich for known BCL6 binding sites in the CXCR4, IL-6 and MCP-1 loci. The y axis represents fold enrichment of binding versus input, as compared with IgG control. Data is represented as the mean ± SEM of three independent experiments. *P < 0.05 vs. DMSO or Saline group.

expressions, the effect of FX1 as a novel BCL6 inhibitor in innate immune response has not been studied. This study firstly investigated how FX1 was able to affect LPS-induced inflammatory responses both in vivo and in vitro. Interestingly, FX1 markedly decreased the lethality of LPS-induced murine sepsis model and was associated with decreased serum levels of pro-inflammatory. We also observed that FX1 treatment attenuated macrophages infiltration in mice lungs and livers and reduced macrophages chemotaxis as evidenced by peritoneal lavage studies. Additionally, FX1 was also shown to be able to decrease production of cytokines and repress BCL6 effects in macrophages in vitro. These findings are the first to showcase the inhibitory properties of FX1 on LPS-induced inflammation and its potential in preventing and treating LPS-induced sepsis through enhancing BCL6 binding affinity to downstream target gene promoters.

Sepsis is a common reason warranting ICU admission and is associated with significant morbidity and mortality [1]. It is well known that sepsis is a systemic inflammatory syndrome caused by a harmful or destructive host response to infection [24–26]. Severe sepsis shows a high mortality rate due to cellular and tissue damage as well as multiple organ failure [3]. Macrophages play a key role in mediating the initial response against pathogens [6], inducing secretion of regulatory pro-inflammatory cytokines such as MCP-1, TNF-α and IL-6 as well as repairing damaged tissues [27–29]. Nevertheless, pro-inflammatory cytokine storms culminate in serious tissue damage, multiple organ failure and death [4]. Based on our knowledge of the inflammatory cascade, inhibiting this detrimental phenomenon remains a potential avenue for controlling inflammatory diseases. Here, we found that FX1 treatment significantly attenuated LPS-stimulated macrophage production of MCP-1, TNF-α, IL-1β and IL-6. IL-1β and TNF-α are the cornerstones of the inflammatory network, working to both induce and maintain the inflammatory response by recruiting and enhancing expressions of other cytokines such as IL-6 [30].

The peritoneal cavity of adult mice contains two populations of macrophages – the small and large peritoneal macrophages (SPM and LPM, respectively). SPM expresses low CD11b and F4/80 levels, while the reverse is true for LPM. The majority of peritoneal macrophages in animals at baseline health are LPM (90%). This population of cells rapidly disappear post-LPS stimulation, with SPM levels increasing after LPS administration. SPM is not derived from LPM but originates from peripheral blood monocytes (MHC class II positive) [22]. This may explain why there is a lower MHC II^+ proportion in SPM after LPS treatment in short time. In addition, MHC class II molecules work in tandem with Toll-like receptor (TLR) 2 and TLR4 to bring about the innate immune response [31,32]. Our research shows that MHC II+ LPMs are significantly increased constitutively, while the SPM colony enlarged after LPS-stimulation. FX1 was able to reverse this change to a certain extent.

LPS-triggered MAPKs cascading activation are critical signal transducers that control inflammatory mediators and cytokines production [33]. In our study, we demonstrate that FX1 inhibits phosphorylation of JNK1/2, p38 MAPK and ERK1/2 in LPS-activated RAW 264.7 cells. Consistent with inflammatory cytokine production TNF-α, IL-1β and IL-6, we suggest that FX1 may repress LPS-induced pro-inflammatory cytokines by at least partially inhibiting the NF-κB signaling pathway.

The MAPK family proteins, particularly ERK, JNK and p38, are regarded as important targets for the development of anti-inflammatory agents [34]. In our study, we demonstrate that FX1 inhibits the phosphorylation of JNK, p38 and ERK in LPS-activated RAW 264.7. Ying et al. showed that BCL6 inhibits Dusp1 expression using a mechanism that depends on DNA binding, which may lead to the modulation of JNK activation and macrophage polarization [35]. This may enable Fx1 to inhibit the phosphorylation of JNK during the early stages after LPS induction. Similar conclusions regarding the P38 and ERK pathways have been obtained using glioblastomas and cardiomyocytes respectively, but not using macrophages [36,37]. In conclusion, the effect of BCL6 on the signaling pathways are activated during the early stages after LPS induction, whereas inflammatory factors are secreted and accumulate during the late stages. Indeed, further mechanistic research needs to be performed.

The significant activation of the canonical TLR4-NF-κB signaling pathway is closely associated with uncontrolled inflammation, tissue damage and septic shock [38]. Components of this critical pathway may potentially be exploited in the development of sepsis-prevention therapy. Our data indicates that BCL6 contributes to the inhibition of the canonical NF-κB pathway in macrophages. Barish et al. have demonstrated the extensive colocalization of BCL6 with NF-kB in BMDMS using ChIP-seq [18]. Furthermore, BCL6 controls inflammation through transcriptional repression via histone deacetylation of inflammatory gene enhancers that are in close proximity to sites inducibly bound by NF-kB and the histone, acetyltransferase p300, following Tlr4 stimulation. Surprisingly, Perez-Rosado et al. found that BCL6 interacts in vivo with the NFκB subunits, RELA/p65 and RELB, of DLBCL-derived cells [17]. Thus, NF-κB activation is a phenomenon that is closely linked with the function of BCL6. We speculate that the capacity of BCL6 to induce NF-κB inhibition is not only dependent on transcriptional repression, but is also possibly associated with protein–protein interaction. However, further confirmation is needed to verify this result.

As a transcriptional repressor, BCL6 represses NF-κB activity and mediates counter-regulation of the innate immune response [16–18]. In addition, BCL6 is able to directly repress its target chemokines and proinflammatory cytokines by specifically binding to the target gene promoters in macrophage [19,20]. However, seemingly contradictory to predecessors' research, we observed that BCL6 specific inhibitor FX1 did not intensify LPS-induced inflammatory response, but instead exhibited an inhibitory effect on inflammatory macrophage activity both in vivo and in vitro. Although BCL6 knockdown has recently been reported to produce a surprising atherosclerosis and xanthomatous tendinitis phenotype [39], $BCL6^{BTBMUT}$ mice did not phenotypically demonstrate lethal $BCL6^{-/-}$ inflammatory disease, indicating that the BTB domain lateral groove is dispensable in order for BCL6 to affect the innate immune system [40]. It was the BCL6 DNA binding domain and not the BTB domain remained the most crucial in inhibiting macrophages chemokine expression. This is the likely explanation as to why FX1 did not aggravate inflammatory response in the phenotype of BCL6-knockout mice, but this does not explain the anti-inflammatory effects of FX1. Therefore, we further investigated by performing ChIP to evaluate the affinity of BCL6 binding domain, and found that the binding of BCL6 to promoters of IL6, CXCR4, MCP-1 were enforced after FX1 treatment. Hence, results presented herein reveal that binding of BCL6 to key macrophage inflammatory genes may be an important biochemical function of BCL6 in innate immunity rather than its interaction with corepressors. Furthermore, we also discovered that 79–6,

the previous generation BCL6 BTB inhibitor [41], also has anti-inflammatory effects in LPS-stimulated macrophages (Supplementary material Fig. S1), though the effects are less than FX1, which to a great extent suggests that blocking the interaction of BCL6 with its corepressors probably strengthens BCL6 binding to the target gene promoters leading to chemokines and proinflammatory cytokine repression.

Mariano G. Cardenas, who discovered FX1, did not provide a proper explanation for the increase in the binding of BCL6 to promoters at the CD69, CXCR4 and DUSP5 loci [21]. The mechanism by which FX1 increases BCL6 binding to promoters remains to be explored. However, Barish et al. demonstrated the extensive colocalization of BCL6 with NF-kB in BMDMS using ChIP-seq [18]. Furthermore, BCL6 controls inflammation through transcriptional repression via histone deacetylation of the inflammatory gene enhancers that are in close proximity to sites inducibly bound by NF-kB and the histone, acetyltransferase p300, following TLR4 stimulation. Thus, the transcriptional repression of BCL6 is closely linked with protein–protein interaction. Accordingly, we speculate that FX1 may have an effect on the protein–protein interactions of BCL6 by occupying part of the BCL6 structural domain, which may lead to an increase of BCL6 binding and transcriptional repression.

In summary, the data presented in this study indicates for the first time that FX1, a novel specific BCL6 BTB inhibitor, is an anti-inflammatory molecule that is able to protect against LPS-induced septic shock. Treatment with FX1 significantly improved sepsis-induced lethality, reduced the production of LPS-induced proinflammatory cytokines, attenuated infiltration of macrophages and chemotaxis of monocytes and decreased the activation of NF-KB through enforcing the binding of BCL6 to target gene promoters. Thus, these results highlight a new role of FX1 as a treatment of sepsis, and that BCL6 may function to be a likely treatment molecule for sepsis. Finally, to some extent, this study is able to eliminate the potential concern from the systemic inflammation caused by the side effects of this kind of drugs. Our data show that toxic side effects of these medications are unlikely, which are in line with previous animal toxicity studies of these inhibitors [42].

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105789.

Statement of ethics

All the animal experimental protocols were approved by the Ethics Committee of Union Hospital, Huazhong University of Science and Technology, China.

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Author contributions

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Analysis and interpretation: PY, JW
Data collection: SC, XD, SC, SL, YZ, HX
Writing the article: HZ, XQ
Critical revision of the article: HZ, XQ
Final approval of the article: PY, JX
Statistical analysis: HZ, XQ, JX
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Overall responsibility: PY, JX.
HZ and XQ contributed equally to this article and share co-first authorship.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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