Journal of Inflammation Research

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

FGF15 Protects Septic Mice by Inhibiting Inflammation and Modulating Treg Responses

Xing Li[1–3], Zexiang Zhu[3], Zhenkun Xia[4], Bo Xu[1,2]

[1]Department of Anesthesiology, The First School of Clinical Medicine, Southern Medical University, Guangzhou, People's Republic of China; [2]Department of Anesthesiology, General Hospital of the Southern Theater Command of the Chinese PLA, Guangzhou, People's Republic of China; [3]Department of Critical Care, Changsha of Traditional Chinese Medicine Hospital, Changsha, People's Republic of China; [4]Department of Thoracic Surgery, The Second Xiangya Hospital of Central South University, Changsha, People's Republic of China

Correspondence: Bo Xu, Tel +86-18907132861, Email xubo333@hotmail.com

Background: Fibroblast growth factor 15 (FGF15) through its FGF-receptor (FGFR)-4 inhibits hepatic inflammation. The current study aimed at investigating whether FGF15 could inhibit septic inflammation and its compensative regulatory T cell (Treg) responses in a mouse sepsis model of cecal ligation and puncture (CLP) and in vitro transwell co-culture.

Methods: Following the sham or CLP procedure, male CLP C57BL/6 mice were intravenously injected with vehicle saline or FGF15 beginning at 2 h post the procedure every 12 h for three days. Some mice were euthanized and their serum and liver samples were collected for examination of cytokines and Tregs by enzyme-linked immunosorbent assay (ELISA), Western blot and flow cytometry. The remaining mice were monitored for their survival up to 14 days post procedure. Moreover, the purified hepatic CD4+ T cells were co-cultured in transwell plates with unmanipulated NCTC 1469 cells or the cells that had been transfected with the control or FGFR4-specific siRNA and treated with, or without, Lipopolysaccharides (LPS) for 24 h, followed by treatment with vehicle PBS or FGF15 for 48 h.

Results: Compared with the CLP group of mice, treatment with FGF15 significantly prolonged the mean survival days of mice (12 vs 1.17 in the CLP group, P = 0.022), mitigated hepatic inflammation and reduced the frequency of apoptotic cells in the liver of mice. FGF15 treatment decreased the percentages of hepatic Tregs, hepatic IL-2, TGF-β and FOXP3 expression in septic mice, accompanied by decreasing serum IL-1β, TNF-α, IL-6 and IL-10 levels. Similarly, FGF15 treatment also attenuated the LPS-increased frequency of Tregs, FOXP3 and IL-2 expression and IL-1β, TNF-α, IL-6 and IL-10 secretion in vitro after co-culture with NCTC 1469 cells, but not co-cultured FGFR4-silenced NCTC 1649 cells.

Conclusion: FGF15 treatment through FGFR4 ameliorated hepatic inflammation and its compensative Treg responses, which were associated with protecting from septic death in mice.

Keywords: FGF15, FGFR4, hepatic inflammation, hepatic Treg infiltrates, septic mice

Introduction

Sepsis is a potentially life-threatening condition caused by host's aberrant responses to infection.[1] In the clinic, the prevalence of sepsis is increasing and sepsis is one of the major factors for high frequency of admission to intensive care units (ICUs).[2] During the process of sepsis, pathogen infection, particularly for gram negative bacterial infection, induces aberrant inflammatory responses by releasing amount of inflammatory mediators, such as pro-inflammatory cytokines of interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNFα) that lead to multiple organ dysfunction and failure.[1,3] Simultaneously, the aberrant pro-inflammatory responses also stimulate compensative anti-inflammatory responses, such as IL-10 and CD4+CD25+FOXP3+ regulatory T cell (Treg) responses to balance and limit the inflammation-induced tissue damages.[4] Despite the available bundled treatments, the mortality of sepsis in ICU patients remains as high as 40% almost.[1] Actually, treatment with blockades of TNFα is insufficient in the control of sepsis and even makes the septic patients susceptible to opportunistic infection.[5–8] Similarly, treatment with anti-IL-6 fails to prolong the survival of septic mice.[9] Hence, discovery of new therapeutic drugs to control inflammation and its associated anti-inflammatory responses may be crucial for intervention of septic patients.

Received: 26 August 2022
Accepted: 1 November 2022
Published: 8 November 2022

The balance of pro-inflammatory and anti-inflammatory responses is critical for homeostasis and control of inflammatory diseases. Pro-inflammatory IL-1β and IL-6 can stimulate naïve T cells to differentiate into T helper 17 (Th17) cells, which facilitate macrophages to secrete more pro-inflammatory cytokines (including of IL-1β, IL-6 and TNFα) and aggravate local tissue inflammation process.[10–13] Similarly, anti-inflammatory cytokines, such as IL-10/TGFβ1 can promote naïve T cell to differentiate into Tregs, which can inhibit inflammation.[14,15] It is notable that fibroblast growth factor 19 (FGF19) is a hormone mainly secreted by ileal epithelial cells under the stimulation of bile acids.[16] Functionally, FGF19 can bind to its high affinity receptor of FGFR4 to regulate bile acid synthesis, glucose metabolism and lipolysis process, as well as many other cellular processes.[17] During the crosstalk between gut and liver inflammation, increased levels of serum FGF19 can inhibit the spreading of intestinal bacteria into the liver portal vein, reducing the severity of hepatic inflammation.[18,19] Similarly, engagement of farnesoid X receptor (FXR) by its ligand, such as bile acid, can induce FGF19 expression and also inhibit the NF-κB signaling-dependent inflammatory cytokine production in hepatocytes.[20,21]

Furthermore, FGF15, an analogue of FGF19 in mice, can reduce adipose deposition and inflammation in the liver, and enhance glucose metabolism, regulating cell survival.[22–25] While excessive hepatic bile acid can lead to hepatocyte inflammation[26] FGF15 over-expression limits hepatic inflammation in a mouse model of nonalcoholic steatohepatitis (NASH).[27] It is interesting that combination of lenvatinib to target FGFR4 and anti-PD-1 (pembrolizumab) enhances antitumor immunity by inhibiting PD-L1 expression and STAT5-related FOXP3 expression as well as Treg infiltration in a mouse model of HCC.[28] These unveil that FGF15 through FGFR4 promotes the development and proliferation of Tregs. However, little is known on whether FGF15 treatment can modulate liver inflammation and its associated Treg responses under a septic condition.

In this study, we tested the hypothesis that treatment with FGF15 can support the survival of septic mice by limiting septic inflammation in the liver and its associated Treg responses in a mouse model of cecal ligation and puncture (CLP)-induced sepsis and the in vitro lipopolysaccharide (LPS)-induced intrinsic toxic cell model.

Materials and Method

A Mouse Model of Sepsis and Treatment

Male C57BL/6 mice (7 weeks old, about 20 grams in body weights) were obtained from STA Laboratory Animal, Changsha, Hunan Province, China and housed in a specific pathogen-free facility with a light/dark cycle of 12 hours at 22~26°C, 50–60% humidity. The mice were allowed to free access to food and water. After adapted in the environment for 7 days, 29 mice were starved for 12 hours and 20 mice were subjected to a procedure of CLP to induce sepsis, as reported previously.[29] The control mice (n = 9) received the sham procedure with cecal ligation, but without cecal puncture and two hours post the procedure, the mice were treated intravenously with saline via the tail vein. Following the CLP procedure, the mice were monitored. Two mice with CLP died within two hours post procedure. The remaining 18 mice were randomized and intravenously injected via the tail vein with vehicle saline (the CLP group) or 400 μg/kg body weight of FGF15 (Abcam, USA) beginning at two hours post the procedure for their recovery every 12 hours for three days (the FGF15 group). All mice were monitored for acting behaviors and survival up to 14 days post procedure and dead mice were recorded.

Another animal experiment was performed using the same protocol and treatment as described above. All mice were euthanized at 72 hours post procedure (n = 9 per group). Their blood samples were collected for preparing serum samples. The mice were anesthetized with 3% isoflurane and transcardially perfused with 100 mL of cooled PBS (0.01 mmol/L, pH7.4). Subsequently, their liver tissues were dissected. Some liver tissues were frozen in liquid nitrogen and stored at −80°C. The remaining liver tissues were fixed in 4% paraformaldehyde, followed by embedded in paraffin. The animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Research and Care Committee of Changsha of Traditional Chinese Medicine Hospital (approval number: No. 2018004).

Histology

The paraffin-embedded liver tissue sections (4~6 μm) were routine-stained with hematoxylin and eosin for microscopy. The pathological changes in individual liver sections were evaluated and scored by experienced pathologists in a blinded manner using the same pathological criteria.[30]

Apoptotic Cells in Mouse Liver Tissues

The impact of FGF15 on the sepsis-induced liver cell apoptosis was determined by TUNEL assay using a TUNEL staining kit, according to the manufacturer's protocol (Roche, USA). The percentages of apoptotic cells in six visual areas selected randomly in individual liver samples were measured for the apoptosis index (AI).

Western Blot Analysis

The frozen liver tissue samples were homogenized in RIPA lysis buffer (Abiowell Biotechnology, Changsha, China) and centrifuged. The liver lysate samples (20 μg per lane) were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10−15% gels, and transferred to nitrocellulose membranes (0.2 μm). After being blocked with 5% non-fat blocking-grade milk in TBST, the membranes were incubated overnight at 4°C with primary antibodies, including anti-FOXP3 (1:1000, Cat. ab215206, Abcam, Cambridge, England), anti-IL-2 (1:1000, Cat. ab207325, Abcam, Cambridge, England), anti-TGF-β (1:1000, Cat. ab215715, Abcam, Cambridge, England), and anti-β-actin (1:1000, Cat. 66009-1-Ig, Proteintech, Rosemont, USA). The bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000, Proteintech, Rosemont, USA) and HRP-goat anti-rabbit IgG (1:6000, Proteintech, Rosemont, USA) and visualized using an enhanced chemiluminescent (ECL) reagent (Abiowell Biotechnology, China). The relative levels of targeted protein to the control β-actin expression were quantified by densitometry analysis using Image J software (Image J 1.51, NIH, USA).

Isolation of Liver CD4+ T Cell Infiltrates

To isolate liver CD4+ T cell infiltrates, individual fresh liver samples were cut into small pieces and digested with 10 mL of 0.25% trypsin (Abiowell Biotechnology, Changsha, China) at 37°C for 1.5 hours, followed by filtration to remove tissue and cell debris. The cell suspensions were centrifuged at 300 g for 10 minutes to pellet the cells and the contained red blood cells were lysed using 5 mL of erythrocyte lysis solution (Abiowell Biotechnology, Changsha, China). The remaining cells were washed three times with PBS solution, and pelleted for isolation of CD4+ T cells using microbeads (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturers' protocol. Briefly, the cell suspensions were incubated with 10 μL of anti-CD4 (TIL) microbeads for 15 minutes in ice and after being washed, the cell suspensions were loaded in a LS column (Miltenyi, Bergisch Gladbach, Germany). The column was washed and the flow-through cells (the absence of CD4+ T cells) were collected. The column-bound CD4+ cells were eluted, centrifuged and suspended in 2 mL of RPMI-1640 (Cat. R8758, Sigma, St Louis, USA).

Flow Cytometric Analysis

The isolated liver CD4+ T cells from individual mice were stained with FITC-labeled anti-CD4 (clone GK1.5, Ebioscience, USA) or APC-labeled anti-CD25 (clone PC61.5, Ebioscience, USA) for 30 minutes in the dark. After being washed, the cells were fixed with Intracellular Fixation buffer (Ebioscience, USA) and permeabilized with Foxp3/Transcription Factor Staining Buffer (Ebioscience, USA), followed by intracellularly staining with PE-labeled anti-FOXP3 (Ebioscience, USA). After being washed, the percentages of CD4+CD25+FOXP3+ Tregs in total CD4+ T cells were analyzed by flow cytometry in a flow cytometry machine (ACEA NovoCyte, USA or Beckman, USA). The data were analyzed by NovoExpressTM software or FlowJo software.

Cell Culture and Transfection

Mouse liver NCTC 1469 cells were obtained from iCell, China and cultured in RPMI-1640 medium supplemented with 10% of fetal bovine serum (FBS, Gibco, USA) in 5% CO_2 incubator at 37°C. NCTC 1469 cells (5×10^4 cells/well) were cultured in 24-well plates overnight and transfected with 5 μL of control si-NC (sequence: 5'-UUCUCCGAACGUGUCACGUTT-3') or FGFR4-specific si-FGFR (sequence:5'-GUCGUACACAAUCUUACGUTT-3') using lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instruction. The efficacy of FGFR4 silencing was tested by Western blot using anti-FGFR4 (1:1000, Cat. Ab178396, Abcam, Cambridge, England).

Co-Culture and Treatment

Because LPS alone usually stimulates the production of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α in hepatocytes,[31] but fail to induce naïve T cell activation and production of cytokines,[32] we co-cultured of NCTC1469 cells with liver T cells to mimic a pathophysiological condition of sepsis to explore the effect of FGF15 treatment on liver inflammation and its compensative Treg responses in vitro.

To determine the potential effect of endotoxin contained FGF15, NCTC 1469 cells (5×10^4 cells/well) were cultured in the upper chambers of 6-well transwell plates (Cat.3450, Corning) and treated with vehicle saline or 200 ng/mL FGF15 (ab206457, Abcam, Cambridge, England) for 24 hours. Subsequently, the cells were co-cultured with the same numbers of hepatic CD4+ T cells from healthy mice in 10% FBS RPMI-1640 in the lower chambers for 96 hours. Their co-culture supernatants were collected for cytokine analysis by ELISA. The T cells in the lower chambers were harvested for analysis of the frequency of Tregs by flow cytometry.

NCTC 1469 cells (5×10^4 cells/well) were cultured in the upper chambers of 6-well transwell plates (Cat.3450, Corning) for 24 hours and the same number of isolated CD4+ T cells were added in the lower chambers in 10% FBS RPMI-1640. The NCTC 1469 cells were stimulated with 100 μg/mL of LPS (Cat.L4516-1mg, Sigma, St Louis, USA) for 24 hours and treated with vehicle saline or 200 ng/mL FGF15 for 48 hours as the LPS and FGF15 groups, respectively. Similarly, the isolated CD4+ T cells were co-cultured in transwell plates with the control si-NCTC1469 or si-FGFR4-NCTC 1469 cells, stimulated with LPS for 24 hours and treated with vehicle saline or the same dose of FGF15 for 48 hours, leading to the si-NC, si-NC+FGF15, si-FGFR4 and siFGFR-FGF15 groups, respectively. At 96 hours post co-culture, their co-culture supernatants were collected for cytokine analysis. The T cells in the lower chambers were harvested for analysis of IL-2, TGF-β, and FOXP3 expression by Western blot and the frequency of Tregs by flow cytometry.

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-1β, TNF-α, IL-6, and IL-10 in the serum samples and co-culture supernatants were quantified by ELISA using specific kits, according to the manufacturer's instructions (Huamei Biotech, Wuhan, China). In the case of IL-1β, the intra-assay coefficient of variation was less than 8%, while the inter-assay coefficient of variance was less than 10%. For other three cytokine assays, both the intra-assay and inter-assay coefficients of variances were the same:less than 10%.

Statistics

Statistical analyses were performed using SPSS version 24.0 for Windows (IBM SPSS, USA). The distributions of quantitative variables in each group were analyzed using the Kolmogorov–Smirnov test. The normally distributed data were expressed as means and standard deviations (SDs), and non-normally distributed data as medians and interquartile ranges (IQRs). The continuous variables between groups were analyzed by ANOVA analysis or the Kruskal–Wallis test where applicable. The categorical variables were compared by the Chi-square test, Fisher's exact test, or McNemar's test where appropriate. The survival of mice was estimated by Kaplan–Meier method and compared by the Log rank test. A P-value of <0.05 was considered statistically significant.

Results

Treatment with FGF15 Prolongs the Survival of Septic Mice by Mitigating Pathological Changes and Apoptosis in the Liver

To explore the potential role of FGF15 in sepsis, C57BL/6 mice were subjected to a CLP procedure, randomized and treated with vehicle saline or FGF15 for three days. There were two mice that died within 2 hours post CLP procedure. The remaining CLP mice and control mice were monitored for their survival up to 14 days post procedure (Figure 1A). While all the mice in the sham group survived for 14 days 2 out of 9 mice in the CLP group survived and 6 out of 9 mice in the FGF15 group remained alive at the end of experiment, leading to a statistical significance in the survival rates ($P = 0.022$). The average survival time of mice in the CLP group was 1.17 days (0.25~14 days) with a death rate of 77.8%, while the average survival period of mice in the FGF15 group was 12 days (1.81~14 days) with 33.3% of death rate. Histological examination revealed that hepatocytes in the CLP group

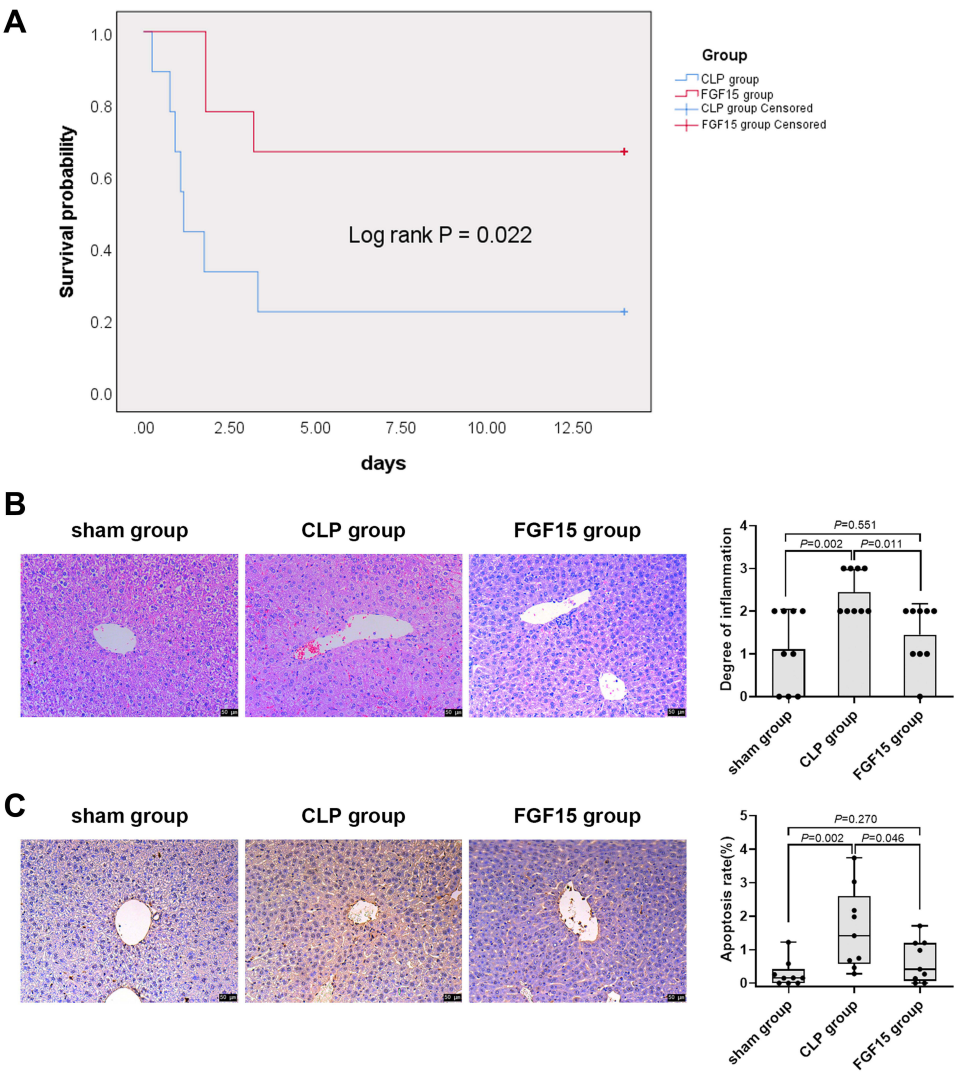

Figure 1 FGF15 treatment supports the survival of septic mice by mitigating hepatic inflammation and liver cell apoptosis. Following the CLP procedure, the mice were randomized and intravenously injected beginning at 2 hours post the procedure every 12 h for three days with saline as the CLP group or FGF15 as the FGF15 group. A sham control group of mice received the sham procedure and saline injection. (**A**) The survival of different groups of mice. (**B**) The histopathological changes in the liver of different groups of mice. (**C**) The percentages of liver cell apoptosis in different groups of mice. Data are representative images or expressed as the mean ± SD of each group (n=9 per group) from three separate experiments.

were enlarged by vacuolar lipid droplets and surrounded by many inflammatory infiltrates. The hepatic sinuses became squeezed and vanished. In contrast, there were obviously less lipid droplets in hepatocytes and inflammatory infiltrates in the liver of mice in the FGF15 group (Figure 1B). Quantitative analysis indicated the inflammatory scores in the CLP group (ranging 2–3) were higher than that in the control and FGF15 groups of mice (ranging 0–2, P = 0.004). TUNEL assays revealed that the frequency of apoptotic cells in the livers of the CLP group of mice was 1.416% (ranging 0.281 ~ 3.738%), which was significantly higher than 0.417% in the FGF15 group (ranging 0.000 ~ 1.223, P = 0.046), and 0.158% in the control group (ranging 0.000 ~ 1.712, P = 0.002, Figure 1C). Hence, treatment with FGF15 significantly supported the survival of CLP mice by mitigating liver inflammation and cell apoptosis.

Treatment with FGF15 Mitigates Inflammation and Treg Infiltrates in the Livers of CLP Mice

To understand the protective role of FGF15, we isolated CD4+ T cell infiltrates from individual liver samples and quantified the frequency of CD4+CD25+FOXP3+ Tregs in individual liver samples by flow cytometry. As shown in Figure 2A, the

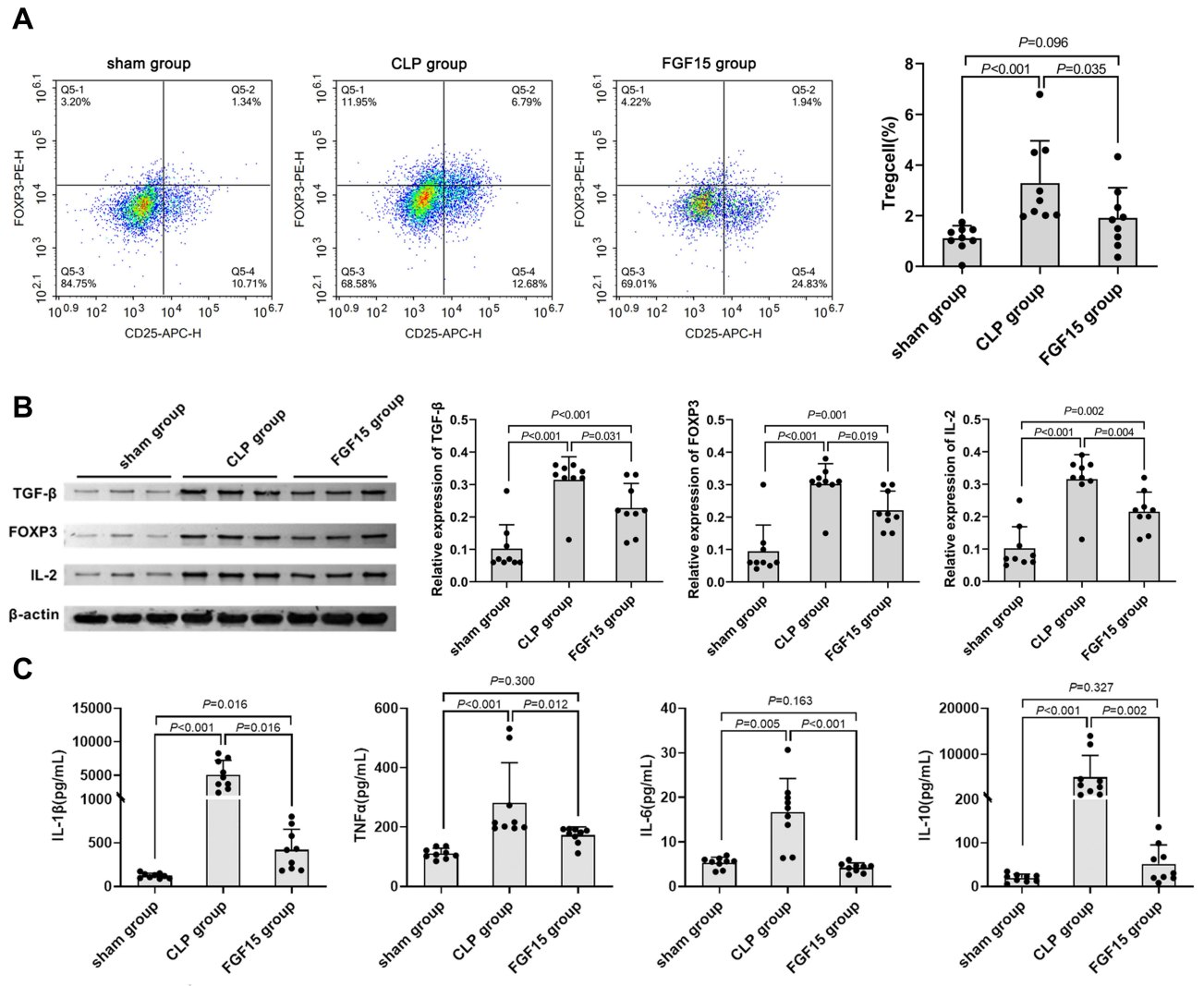

Figure 2 FGF15 treatment mitigates hepatic Treg responses and reduces the levels of serum cytokines in septic mice at 3 days post CLP. (**A**) Flow cytometric analysis of the frequency of hepatic CD4+CD25+FOXP3+ Tregs in total CD4+ T cells in different groups of mice. (**B**) Western blot analysis of the relative levels of IL-2, FOXP3 and TGF-β expression in the liver of mice. (**C**) ELISA analysis of the levels of serum cytokines in different groups of mice. Data are representative images or expressed as the mean ± SD of each group (n=9 per group) from three separate experiments.

median percentages of hepatic Tregs in the CLP group (2.60, 1.97~6.79%) were significantly higher than that in the control group (1.13, 0.04~1.74%, $P < 0.001$) and in the FGF15 group (1.84, 0.36~4.33%, $P = 0.035$). There was no significant difference in the frequency of hepatic CD4+CD25+FOXP3+ Tregs between the FGF15 and the control groups of mice ($P = 0.096$). Western blot analysis indicated similar patterns of the relative levels of hepatic IL-2, TGF-β and FOXP3 protein expression among the groups of mice (Figure 2B). Moreover, analysis of circulating cytokines revealed that compared with the control group, the levels of serum IL-1β, IL-6, TNF-α and IL-10 in the CLP group were significantly elevated, which were dramatically reduced in the FGF15 group of mice (Figure 2C). Thus, treatment with FGF15 significantly mitigated the CLP-stimulated inflammation and inflammation-compensated Treg responses in the liver of mice.

Treatment with FGF15 Inhibits the LPS-Stimulated Inflammation and Compensative Treg Responses in the Co-Cultured NCTC 1469 Cells with Hepatic CD4+ T Cell Infiltrates in vitro

Next, we tested whether FGF15 we used could modulate the production of inflammatory cytokines and its compensated Treg responses in a cellular model by co-culture of mouse NCTC 1469 hepatocytes with CD4+ T cells from the liver of healthy mice.

The results revealed that the levels of IL-6, IL-1β, TNF-α and IL-10 in the supernatants of co-cultured cells were not statistically different between the control and FGF15 groups (Supplementary Table 1). Furthermore, there was also no significant difference in the frequency of hepatic CD4+CD25+FOXP3+ Tregs between the FGF15 group (0.56 ± 0.18%) and the control group (0.38 ± 0.18%, P = 0.286, Supplementary Figure 1). These suggest that the FGF15 we used may contain little endotoxin and have little role in regulating the spontaneous production of those cytokines in mouse hepatocytes in our experimental conditions.

To further understand how liver cells affected Treg responses under an inflammatory condition, we co-cultured hepatic CD4+ T cell infiltrates with control or FGFR4-silenced NCTC 1469 cells. After stimulation with the vehicle control or LPS for 24 hours and treatment with, or without, FGF15 for another 48 hours, we analyzed the frequency of Tregs in different groups of cells by flow cytometry. Compared with the control cells, the percentages of Tregs in the LPS group significantly increased

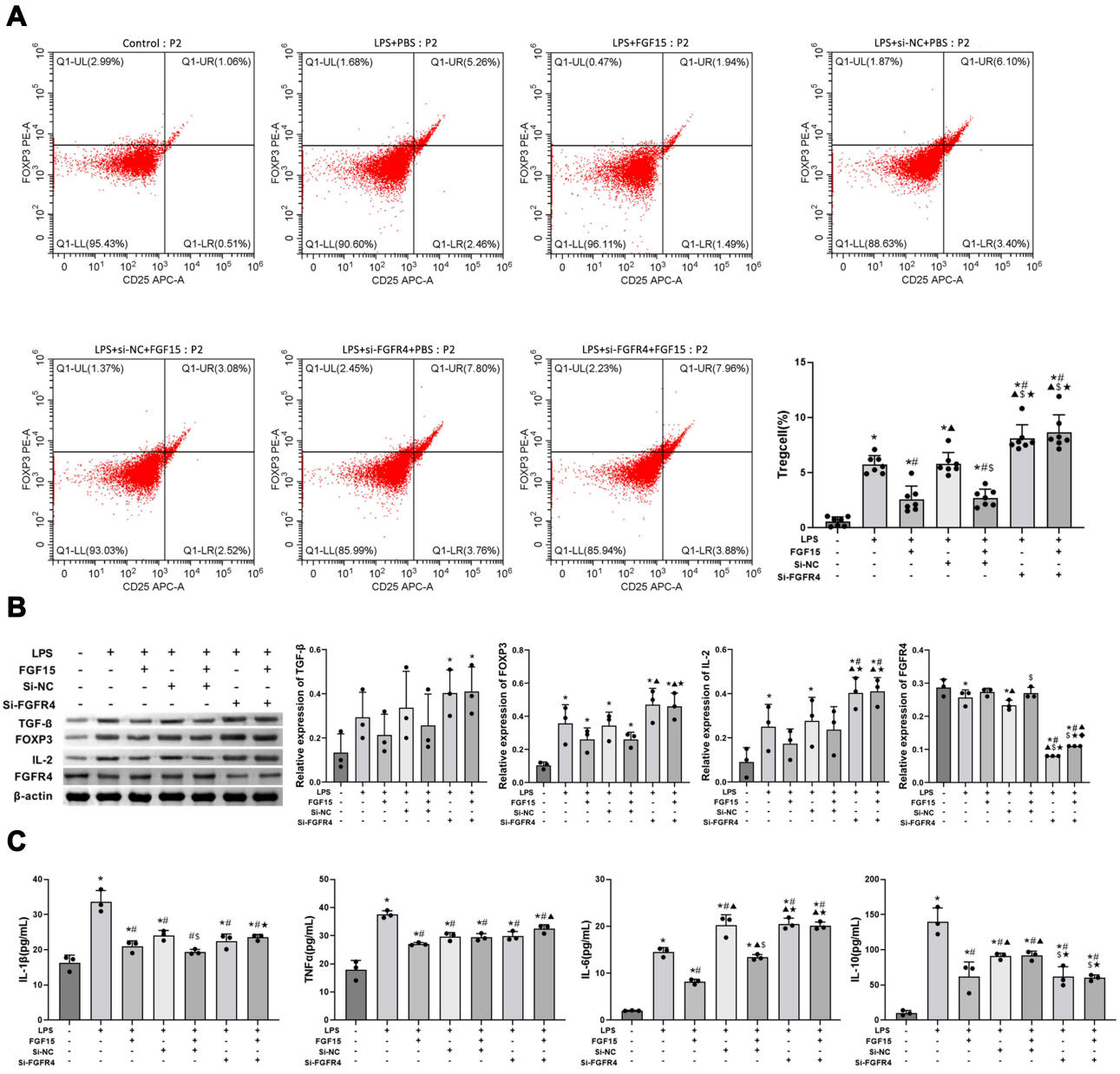

Figure 3 FGF15 treatment mitigates the LPS-enhanced Treg responses in a transwell co-culture system. Liver NCTC 1469 cells were transfected with control or FGFR4-specific siRNA and co-cultured with the isolated liver CD4+ T cells from naïve mice in transwell plates. The cells were stimulated with LPS for 24 h and treated with vehicle saline or FGF15 for 48 h. (**A**) Flow cytometry analysis of the frequency of CD4+CD25+FOXP3+ Tregs in total CD4+ T cells. (**B**) Western blot analysis of the relative levels of FGFR4, TGF-β and IL-2 expression in different groups of cells. (**C**) ELISA analysis of the levels of IL-1β, TNFα, IL-6 and IL-10 in the supernatants of cultured cells. Data are representative images or expressed as the mean ± SD of each group of cells from three separate experiments. $^{*}P < 0.05$ vs the control group; $^{\#}P < 0.05$ vs the LPS group; $^{\blacktriangle}P < 0.05$ vs the FGF15 group; $^{\$}P < 0.05$ vs the LPS+Si-NC group; $^{\bigstar}P < 0.05$ vs the LPS+Si-NC+FGF15 group; $^{\blacklozenge}P < 0.05$ vs the LPS+Si-FGFR4 group.

(5.74±0.31%, P = 0.000), which were significantly reduced in the FGF15-treated cells (2.60±0.45%, P = 0.000, Figure 3A). Interestingly, the percentages of Tregs in the si-FGFR4 group were further higher (8.11± 0.47%) than that in the LPS group and the FGF15 group (P = 0.000). However, treatment with FGF15 failed to significantly decrease the frequency of Tregs in the si-FGFR4 group of cells. These indicated that FGF15 treatment through its FGFR4 inhibited the inflammation-stimulated compensative Treg responses. Western blot revealed similar patterns of the relative levels of IL-2 and FOXP3 protein expression among the different groups of T cells and significantly decreased FGFR4 expression (about 65%) in hepatocytes (Figure 3B). Compared with normal cultured cell group, the relative levels of FOXP3 expression were much higher in all the LPS stimulation groups. Interestingly, when comparing with FGF15-treated groups (LPS+FGF15 and LPS+Si-NC+FGF15 groups), both the relative levels of FOXP3 and IL-2 expression significantly increased in the Si-FGFR4 groups (LPS+Si-FGFR4+PBS and LPS+Si-FGFR4+FGF15 groups). Finally, we analyzed the levels of inflammatory cytokines in the supernatants of co-cultured cells by ELISA (Figure 3C). Compared with the control cells, LPS stimulation significantly increased the levels of IL-6, IL-1β, TNF-α, and IL-10 production by the co-cultured cells, mainly by hepatocytes, but the levels of IL-1β, IL-6, TNF-α and IL-10 were all significantly reduced by FGF15 treatment. While FGFR4 silencing failed to modulate the LPS-stimulated IL-6, IL-1β, TNF-α production it did reduce IL-10 production in the LPS-treated FGFR4-silenced cells. Treatment with FGF15 also did not alter the levels of IL-6, IL-1β, TNF-α, and IL-10 production in the FGFR4-silenced cells. Therefore, treatment with FGF15 inhibits the LPS-stimulated inflammation and compensative Treg responses in the co-cultured NCTC 1469 cells with hepatic CD4+ T cell infiltrates in vitro in a FGFR4-dependent manner.

Discussion

In this study, our findings highlighted that 1) intravenous treatment with FGF15 for a short period protected from sepsis-related death in mice by significantly inhibiting hepatic inflammation and cell apoptosis; 2) Treatment with FGF15 also mitigated the inflammation-related compensative hepatic Treg responses, decreased the levels of hepatic TGF-β, IL-2 and FOXP3 expression and reduced the serum levels of both pro-inflammatory IL-1β, TNF-α, IL-6 and anti-inflammatory IL-10 in septic mice; and 3) treatment with FGF15 attenuated the LPS-induced inflammation, cytokine secretion and the inflammation-compensative Treg responses in a transwell co-culture system in a FGFR4-dependent manner. Such novel findings suggest that FGF15 may be valuable for the intervention of septic liver damages.

Compared with the sham controls, the CLP procedure induced severe inflammation in the liver of septic mice. Evidently, high levels of circulating IL-1β, TNF-α, IL-6 and IL-10 and many inflammatory infiltrates in the liver were detected in septic mice, accompanied by moderate levels of liver cell apoptosis, which may contribute to a high rate of death. These demonstrated that the CLP procedure resulted in severe bacterial infection and sepsis, leading to liver damages and death.[33] Interestingly, we observed a high frequency of hepatic Treg infiltrates in septic mice. Our data were consistent with previous observations,[34,35] and extending previous findings of high percentages of Tregs in the spleen and lung of septic animals.[36–38] It is well known that Tregs can secrete anti-inflammatory IL-10 and TGF-β as well as other inhibitor mediators to inhibit inflammation. Hence, during the septic process, severe inflammation can compensatively up-regulate Treg responses by recruiting Treg infiltrates and enhancing their proliferation in the inflammatory lesions to limit and control inflammation and tissue damages. Notably, severe inflammation enhances high levels of IL-2 secretion, which acts its receptors and STAT5 signaling to promote the proliferation of nature and inducible Tregs during the early stage of septic process.[39]

More importantly, treatment with FGF15 significantly mitigated systemic and hepatic inflammation, liver cell apoptosis and Treg responses to protect from death in septic mice. Similarly, FGF15 treatment also reduced the frequency of Tregs in CD4+ T cells that had been co-cultured with the LPS-treated hepatocytes and decreased their FOXP3 expression and IL-6, IL-1β, TNF-α, and IL-10 secretion in a FGFR4-dependent manner in vitro. It is possible that FGF15 through its FGFR4 may inhibit the pro-inflammatory cytokine responses and down-regulate the IL-2/STAT5 signaling to attenuate the proliferation of nature and inducible Tregs, reducing the frequency of Tregs in CLP livers of mice. The simultaneous attenuation of hepatic inflammation and Treg responses supports the notion that the balance of pro-inflammatory and anti-inflammatory responses is crucial for the control of septic liver injury.[40] These results were consistent with our previous clinical study, and the serum levels of FGF19 were much higher in the non-survival septic patients compared with survival septic patients.[41]

FGFR4 is widely expressed on the surface of liver epithelial cells as well as some of other organs.[22] Previous studies have shown that FGF15 through its FGFR4 can enhance glucose metabolism and promote lipid oxidation.[23,24] In this study, we found that FGF15 treatment inhibited the LPS-stimulated inflammation and compensative Treg responses in a FGFR4-dependent manner in the co-cultured NCTC 1469 cells with hepatic CD4+ T cell infiltrates. In contrast, FGFR4 silencing in liver cells significantly increased the frequency of Tregs after the co-cultured CD4+ T cells. These suggest that FGF15 should first bind to the FGFR4 on hepatocytes and reduce the LPS-stimulated inflammation in hepatocytes and the inflammation-compensated Treg responses. Thus, activation of the FGFR4 may be a therapeutic strategy to inhibit hepatic inflammation and its compensative Treg responses.

We recognized that our study had limitations. Firstly, in this study, we treated the mice with FGF15 and generated FGFR4 silencing liver cells so that we can not exclude any potential effect of endogenous FGFR4 agonist on inflammation and Treg responses. Secondly, sepsis can affect many other organs, besides the liver, but we only centered on the liver inflammation and Treg responses. Thirdly, due to the limited resource of mouse liver tissues, we did not measure Th17 responses and other cellular immune responses in the liver, which may be important for the pathogenesis of septic liver damages. Furthermore, we did not directly quantify the contents of endotoxin in the used FGF15 although we provided indirect evidence of little endotoxin in the used FGF15 in vitro. Finally, we did not analyze whether FGFR4 was expressed by CD4+ T cell and Tregs and explore the relative levels of FGFR4 phosphorylation due to the lack of specific antibody although we recognized the FGFR4 phosphorylation might be crucial for the affinity of FGF15 binding and FGFR4 expression would be important for understanding the indirect or direct effect of FGF15 on Tregs. We are interested in further investigating how knockout of both FGF15 and FGFR4 can modulate the inflammation, Th17 responses and inflammation-compensative Treg responses in different organs.

Conclusions

Our findings indicated that FGF15 treatment through its FGFR4 protected from septic liver damages and death in mice, which were associated with attenuating inflammation and its compensative Treg responses.

Data Sharing Statement

The authors confirm that the data supporting the findings of this study are available within the article.

Ethics Approval

The study was approved by the Ethics Committee of Changsha of Traditional Chinese Medicine Hospital (No. 2018004).

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; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the National Natural Science Foundation of China (Grant No.62076253); Natural Science Foundation of Guangdong Province, China (Grant No.2021A1515010077); and Natural Science Foundation of Hunan Province, China (Grant No.2019JJ80061).

Disclosure

The authors declare that there are no conflicts of interest.

References

1. Singer M, Deutschman CS, Seymour CW, et al. The third international consensus definitions for sepsis and septic shock (Sepsis-3). *JAMA*. 2016;315(8):801–810. doi:10.1001/jama.2016.0287
2. Merino I, de la Fuente A, Domínguez-Gil M, Eiros JM, Tedim AP, Bermejo-Martín JF. Digital PCR applications for the diagnosis and management of infection in critical care medicine. *Crit Care*. 2022;26(1):63. doi:10.1186/s13054-022-03948-8
3. Doran KS, Banerjee A, Disson O, Lecuit M. Concepts and mechanisms: crossing host barriers. *Cold Spring Harb Perspect Med*. 2013;3(7): a010090. doi:10.1101/cshperspect.a010090
4. Tamayo E, Fernández A, Almansa R, et al. Pro- and anti-inflammatory responses are regulated simultaneously from the first moments of septic shock. *Eur Cytokine Netw*. 2011;22(2):82–87. doi:10.1684/ecn.2011.0281
5. Dinarello CA. How interleukin-1β induces gouty arthritis. *Arthritis Rheum*. 2010;62(11):3140–3144. doi:10.1002/art.27663
6. Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol*. 2011;11(2):98–107. doi:10.1038/nri2925
7. Solovic I, Sester M, Gomez-Reino JJ, et al. The risk of tuberculosis related to tumour necrosis factor antagonist therapies: a TBNET consensus statement. *Eur Respir J*. 2010;36(5):1185–1206. doi:10.1183/09031936.00028510
8. Grau GE, Maennel DN. TNF inhibition and sepsis – sounding a cautionary note. *Nat Med*. 1997;3(11):1193–1195. doi:10.1038/nm1197-1193
9. Barkhausen T, Tschernig T, Rosenstiel P, et al. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Crit Care Med*. 2011;39(6):1407–1413. doi:10.1097/CCM.0b013e318211ff56
10. Peterson LW, Artis D. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol*. 2014;14(3):141–153. doi:10.1038/nri3608
11. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. 2006;24(2):179–189. doi:10.1016/j.immuni.2006.01.001
12. Yao Z, Painter SL, Fanslow WC, et al. Human IL-17: a novel cytokine derived from T cells. *J Immunol*. 1995;155(12):5483–5486.
13. Erbel C, Akhavanpoor M, Okuyucu D, et al. IL-17A influences essential functions of the monocyte/macrophage lineage and is involved in advanced murine and human atherosclerosis. *J Immunol*. 2014;193(9):4344–4355. doi:10.4049/jimmunol.1400181
14. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006;441(7090):235–238. doi:10.1038/nature04753
15. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol*. 2004;22:531–562. doi:10.1146/annurev.immunol.21.120601.141122
16. Song KH, Li T, Owsley E, Strom S, Chiang JY. Bile acids activate fibroblast growth factor 19 signaling in human hepatocytes to inhibit cholesterol 7alpha-hydroxylase gene expression. *Hepatology*. 2009;49(1):297–305. doi:10.1002/hep.22627
17. Tomlinson E, Fu L, John L, et al. Transgenic mice expressing human fibroblast growth factor-19 display increased metabolic rate and decreased adiposity. *Endocrinology*. 2002;143(5):1741–1747. doi:10.1210/endo.143.5.8850
18. Chen WLK, Edington C, Suter E, et al. Integrated gut/liver microphysiological systems elucidates inflammatory inter-tissue crosstalk. *Biotechnol Bioeng*. 2017;114(11):2648–2659. doi:10.1002/bit.26370
19. Inagaki T, Moschetta A, Lee YK, et al. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci U S A*. 2006;103(10):3920–3925. doi:10.1073/pnas.0509592103
20. Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W. Farnesoid X receptor antagonizes nuclear factor kappaB in hepatic inflammatory response. *Hepatology*. 2008;48(5):1632–1643. doi:10.1002/hep.22519
21. Inagaki T, Choi M, Moschetta A, et al. Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab*. 2005;2(4):217–225. doi:10.1016/j.cmet.2005.09.001
22. Tomiyama K, Maeda R, Urakawa I, et al. Relevant use of Klotho in FGF19 subfamily signaling system in vivo. *Proc Natl Acad Sci U S A*. 2010;107(4):1666–1671. doi:10.1073/pnas.0913986107
23. Huang X, Yang C, Luo Y, Jin C, Wang F, McKeehan WL. FGFR4 prevents hyperlipidemia and insulin resistance but underlies high-fat diet induced fatty liver. *Diabetes*. 2007;56(10):2501–2510. doi:10.2337/db07-0648
24. Kir S, Beddow SA, Samuel VT, et al. FGF19 as a postprandial, insulin-independent activator of hepatic protein and glycogen synthesis. *Science*. 2011;331(6024):1621–1624. doi:10.1126/science.1198363
25. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer*. 2010;10(2):116–129. doi:10.1038/nrc2780
26. Arab JP, Karpen SJ, Dawson PA, Arrese M, Trauner M. Bile acids and nonalcoholic fatty liver disease: molecular insights and therapeutic perspectives. *Hepatology*. 2017;65(1):350–362. doi:10.1002/hep.28709
27. Matye DJ, Wang H, Luo W, et al. Combined ASBT Inhibitor and FGF15 treatment improves therapeutic efficacy in experimental nonalcoholic steatohepatitis. *Cell Mol Gastroenterol Hepatol*. 2021;12(3):1001–1019. doi:10.1016/j.jcmgh.2021.04.013
28. Yi C, Chen L, Lin Z, et al. Lenvatinib targets FGF receptor 4 to enhance antitumor immune response of anti-programmed cell death-1 in HCC. *Hepatology*. 2021;74(5):2544–2560. doi:10.1002/hep.31921
29. Baker CC, Chaudry IH, Gaines HO, Baue AE. Evaluation of factors affecting mortality rate after sepsis in a murine cecal ligation and puncture model. *Surgery*. 1983;94(2):331–335.
30. Klopfleisch R. Multiparametric and semiquantitative scoring systems for the evaluation of mouse model histopathology--a systematic review. *BMC Vet Res*. 2013;21(9):123. doi:10.1186/1746-6148-9-123
31. Gaitantzi H, Karch J, Germann L, et al. BMP-9 Modulates the Hepatic Responses to LPS. *Cells*. 2020;9(3):617. doi:10.3390/cells9030617
32. Reynolds JM, Martinez GJ, Chung Y, Dong C. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. *Proc Natl Acad Sci*. 2012;109(32):13064–13069. doi:10.1073/pnas.1120585109
33. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? *Trends Microbiol*. 2011;19(4):198–208. doi:10.1016/j.tim.2011.01.001
34. Yeh CL, Tanuseputero SA, Wu JM, et al. Intravenous arginine administration benefits CD4+ T-cell homeostasis and attenuates liver inflammation in mice with polymicrobial sepsis. *Nutrients*. 2020;12(4):1047. doi:10.3390/nu12041047
35. Di Caro V, Cummings JL, Alcamo AM, et al. Dietary cellulose supplementation modulates the immune response in a murine endotoxemia model. *Shock*. 2019;51(4):526–534. doi:10.1097/SHK.0000000000001180

36. Shen X, Li N, Li H, Zhang T, Wang F, Li Q. Increased prevalence of regulatory T cells in the tumor microenvironment and its correlation with TNM stage of hepatocellular carcinoma. *J Cancer Res Clin Oncol*. 2010;136(11):1745–1754. doi:10.1007/s00432-010-0833-8
37. Zhao J, Liu Y, Hu JN, et al. Autocrine regulation of interleukin-3 in the activity of regulatory T cells and its effectiveness in the pathophysiology of sepsis. *J Infect Dis*. 2021;223(5):893–904.
38. Tran DT, Jeong YY, Kim JM, Bae HB, Son SK, Kwak SH. The anti-inflammatory role of bilirubin on "Two-Hit" sepsis animal model. *Int J Mol Sci*. 2020;21(22):8650. doi:10.3390/ijms21228650
39. Osinalde N, Mitxelena J, Sánchez-Quiles V, et al. Nuclear phosphoproteomic screen uncovers ACLY as mediator of IL-2-induced proliferation of CD4+ T lymphocytes. *Mol Cell Proteomics*. 2016;15(6):2076–2092. doi:10.1074/mcp.M115.057158
40. Elenkov IJ, Iezzoni DG, Daly A, Harris AG, Chrousos GP. Cytokine dysregulation, inflammation and well-being. *Neuroimmunomodulation*. 2005;12(5):255–269. doi:10.1159/000087104
41. Li X, Zhou T, Zhu Z, Xu B. High concentration of serum FGF19 at ICU admission is associated with 28-day mortality in sepsis patients. *Clin Chim Acta*. 2021;523:513–518. doi:10.1016/j.cca.2021.11.002