Plasma fibronectin can affect the cytokine profile and monocytes/macrophages function in addition to predicting the prognosis of advanced sepsis

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Abstract
The value of plasma fibronectin (pFN) in the diagnosis and prognosis of sepsis has not been fully established. Previous studies finding that pFN is significantly reduced in sepsis, however, whether reduced pFn affects the prognosis of sepsis has not been clarified. Here, we detected and analyzed pFN and other conventional inflammatory markers in advanced sepsis patients and performed correlation analysis with SOFA score. We also used Fn gene conditional knockout mice which were performed by cecum ligation and puncture (CLP) to investigate the effect of FN deficiency on sepsis prognosis. We found, compared with procalcitonin, c-reactive protein, and interleukin-6, pFN was more correlated with SOFA score in advanced sepsis patients (\( r = -0.720, p < .001 \)). In animal experiments, Fn gene knockout mice showed significantly greater mortality after CLP compared with the control group because of inhibited phagocytosis and bacterial clearance ability of macrophages, with double cytokine storm. Furthermore, FN can regulate macrophages through the integrin \( \alpha_5\beta_1/Fak/Src \) signaling pathway. Overall, we found pFN can more accurately reflect the severity and prognosis of advanced sepsis. The absence of FN altered the cytokine storm and phagocytic function of macrophages, suggesting that FN could be a potential therapeutic target in sepsis.

KEYWORDS
cytokine storm, fibronectin, immunosuppression, macrophage, sepsis
1 | INTRODUCTION

Sepsis or septic shock is still a major clinical cause of death and the most important clinical problem in acute critical care medicine. Sepsis affects more than 19 million people worldwide each year, with a case fatality rate of over 30%. Currently, the global epidemic of COVID-19 is aggravating the incidence of sepsis. It has been clarified that the two peak periods of death in patients with sepsis are early immune over activation (cytokine storm) and late sustained immunosuppression, respectively. With the intervention of effective antibiotics and the improvement of adjuvant and supportive treatment, early mortality of sepsis has been improved. Nonetheless, there are still some patients who are at risk of death due to the continued immunosuppression in the later stage of sepsis. Consequently, ongoing research is attempting to regulate the immune status of patients with sepsis, control cytokine storm, reduce the probability of secondary infection, and promote the repair of damaged tissue, thereby improving the prognosis of patients with sepsis.

Fibronectin (FN) is an important adhesion molecule widely distributed in the interstitial space with multiple functional domains and participate in cell adhesion, migration, proliferation, and differentiation. It was called CIG (cold-insoluble globulin) before being named as fibronectin. As early as the 1980s, Eriksen HO et al. reported the changes of plasma FN (pFN) in patients with sepsis and severe burns in intensive care unit for the first time, suggesting that survival of these patients was related to the extent of decrease in the pFN concentration. Follow-up researchers found that pFN was significantly reduced in patients with severe infection, and the degree of pFN reduction could reflect the severity of sepsis, so pFN may be a potential target for the diagnosis and treatment of sepsis. In 2001, Fässler et al. established the Fn gene conditional knockout mice based on the cyclo-recombinase (Cre-loxP) system. The effects of pFN on neuronal injury repair, skin injury repair, coagulation, and atherosclerosis were systematically studied. It is proved that pFN plays a self-limiting regulatory role in thrombosis and platelets play important roles in sepsis and blood coagulation. However, no reports are available so far on the effects of Fn gene knockout on infection and immune regulation. To the best of our knowledge, the role and detailed mechanism of FN in regulating infection immunity have not been clarified, the value of pFN in evaluating the prognosis of sepsis has yet to be translated into clinical application. So far, little is known about the function of pFN in regulating inflammation and immunity, especially in the immune regulation of sepsis. The sepsis mortality in Fn knockout (Fn KO) mice has not been reported.

The greatest advantage of FN is that it can be used as an alternative therapeutic target. Previously, we successfully expressed two heparin-bound domain functional peptides at the n-terminal and c-terminal of fibronectin using genetic engineering technology. We also demonstrated that the recombinant fibronectin polypeptide antagonizes hepatic failure induced by endotoxin in mice, these polypeptides were shown to improve the prognosis of sepsis as well as inhibit liver cancer cell metastasis in mice. In order to further clarify the value of pFN in the diagnosis and treatment of sepsis, we detected and analyzed pFN and other conventional inflammatory markers in advanced sepsis patients and performed correlation analysis with SOFA score in this study. We also used Fn gene conditional knockout mice which were performed by cecum ligation and puncture (CLP) to investigate the effect of FN deficiency on sepsis prognosis, showing that the absence of FN had an important effect on the mortality of mice with sepsis. This study will reposition the value of pFN in evaluating the prognosis of sepsis and elucidate the mechanism by which FN affects the prognosis of sepsis in murine model.

2 | MATERIAL AND METHODS

2.1 | Clinical specimens

The collection of all human plasma samples was approved by the Ethical Commission of the Fujian Medical University Union Hospital and written informed consent was obtained from all subjects. Plasma samples of healthy, infections, and septic patients were collected from the healthy population in the physical examination center and inpatients in the intensive care department of Fujian Medical University Union Hospital, China, respectively. Infections (bacterial infections) were diagnosed based on clinical manifestation, laboratory results, recovery of pathogens, and radiologic studies. The diagnosis of bacterial infection was defined as positive microbial culture results of body fluids including blood, sputum, urine, pleural fluid, peritoneal fluid, and cerebrospinal fluid among patients. The diagnostic criteria of sepsis were according to the definition and diagnostic criteria of sepsis 3.0, jointly issued by the American Critical Care Medical Association and European Critical Care Medical Association in 2016. We define advanced sepsis as one that lasts more than 1 week in clinical practice, and early sepsis as one that lasts no more than 1 week after diagnosis. Sequential (sepsis-related) organ failure assessment (SOFA) score were calculated, analyzed, and correlated with procalcitonin (PCT), C-reactive protein (CRP), interleukin-6 (IL-6), and plasma FN.
2.2 Animals

C57BL/6J mice, 8 to 12 weeks old, specific pathogen-free, weighing approximately 22 g were purchased from Shanghai SLAC LABORATORY ANIMAL COMPANY. Fn Flox (Fn loxP+/+) mice and UBC-cre/ERT2 mice which were on a C57BL/6 genetic background were generated with the help of Shanghai Biomodel Organism Science & Technology Development.32 Mice were bred and maintained under specific pathogen-free conditions. All animals were handled in strict accordance with good animal practice as defined by the National Regulations for the Administration of Experimental Animals of China. Animal protocols and experimental procedures were also approved by the Institutional Animal Care and Use Ethics Committee of the Fujian Medical University.

2.3 Sepsis models

Sepsis was induced by a modified cecal ligation and puncture (CLP) in mice aged 8 to 12 weeks old.33 CLP was performed in a modified operative method, briefly, a 1.5 cm length of the cecum was ligated and punctured once with a 22-gauge needle in the middle, the cecum was then gently squeezed to extrude small amounts of feces from the perforation sites before the ligated and punctured cecum was placed back into the abdomen. Peritoneum was closed with 6.0 silk sutures, followed by resuscitation with 1 ml warm saline. Mice were kept warm at the blanket for 1 h after surgery. Tramadol was given twice to relieve pain at 3 and 12 h after surgery. In the recovery experiment, Fn KO mice were injected with FN (20 mg or 10 mg/kg, q8h*3 days, and the first dose was administered 2 h before CLP) or the same volume of normal saline in the tail vein. Mice were monitored twice daily by personnel experienced in recognizing signs of a moribund state. Mice were observed for 20 days after operation as endpoint.

2.4 Bacterial load, phagocytosis, bacterial killing, and colony-forming unit assay

Mice were euthanized at 6 h time points after CLP, cardiac puncture was performed, the blood was subjected to 10–50 fold dilution with sterile PBS, 50 μl of each sample was applied to 5% sheep blood agar which was counted overnight at 37°C for colony-forming units (CFUs) formation test. Escherichia coli (ATCC25922) and Enterococcus faecalis (ATCC29212) were cultured in LB liquid and adjusted to appropriate concentration. To determine ability of peritoneal macrophages clear bacteria, mice were euthanized at 6 and 24 h after intraperitoneal injection of E. coli (OD0.4, 200 μl per mouse) and E. faecalis (OD 0.2, 200 μl per mouse), the peritoneal lavage fluid was collected by washing the peritoneal cavity with 3 ml sterile PBS, after 10-fold dilution, 100 μl of each sample was applied to sheep blood agar for overnight bacterial CFU formation at 37°C. E. coli–FITC were made in our own laboratory, peritoneal macrophages from WT and Fn KO mice were infected with E. coli–FITC (1:10) in HBSS with 20% serum, which was from their own or control mice. Incubated at 37°C for 15, 30, and 40 min for the phagocytosis assay by flow cytometry (FCM). RAW264.7 cells were infected with E. coli–FITC (1:10) in serum-free HBSS under Fn0, Fn50, and Fn100 μg/ml for 30 min and the bacterial content in cells was determined by a fluorescence microplate reader.

2.5 Flow cytometry

Cells were blocked for Fc receptors with anti-mouse CD16/32 (BD Pharmingen) for 10 min and then were stained with fluorochrome-conjugated antibody for 30 min. To identify the monocytes/macrophages, mouse blood and peritoneal lavage fluid were labeled with PE-CD11b (BD Pharmingen) and CD86 (BD Pharmingen) for M1-type, PE-CD11b, and CD206 (BD Pharmingen) for M2-type. Data were acquired with a BD FACS Verse flow cytometer (BD Bioscience) and analyzed with FlowJo 10 analytical software.

2.6 Assessment of cytokines and plasma FN

Human plasma PCT, CRP, IL-6, and FN were measured by ELISA kits (Abcam). Mice plasma samples were analyzed using the Bio-Plex Pro Mouse CytokineGrp1 Panel 23-plex (R&D Systems) contain interleukin 1 (IL-1α, IL-1β), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, eotaxin, G-CSF, GM-CSF, IFN-γ, KC/CXCL1, MCP-1, MIP-1α, MIP-1b, RANTES, and TNF-α. IL-1α, IL-6, and TNF-α in cell culture supernatants were quantified with ELISA kits (BD or R&D systems). Nitric oxide (NO) concentration was quantified according to the manufacturer’s instructions (Solarbio). Mouse plasma FN were measured by ELISA kits (Abcam).

2.7 Isolation of primary macrophages and cell culture

Mice were euthanized by pentobarbital anesthesia, peritoneal cavity was washed with 5 ml cool sterile PBS
containing 1% fetal bovine serum (FBS, PAN) for twice. After centrifugation at 800 rpm for 5 min at 4°C, the cell pellet was re-suspended in 1 ml sterile PBS, and the total cell number was counted under microscopy. The cell concentration was adjusted to 2 × 10^5/ml and incubate in six-well plates for 2 h with RPMI 1640 medium containing 10% FBS, and with medium to wash non-adherent cells. The resulting macrophage monolayer was 96% pure and ready for experimentation. The murine macrophage cell line RAW264.7 (SCSP-5036) was obtained from the Chinese Academy of Sciences Shanghai Cell Bank and cultured in DMEM High Glucose containing 10% FBS (PAN) with penicillin and streptomycin (HyClone). RAW264.7 cells were cultured at different concentrations of FN (0, 25, 50, and 100 µg/ml) (CORNING), which were stimulated by LPS (1 µg/ml) (Sigma-Aldrich).

2.8 | Quantitative RT-PCR

Total RNA was extracted with the Simply Total RNA Extraction Kit (BioFlux) according to the manufacturer’s instructions. Luna-one step, quantitative real-time reverse-transcription PCR (RT-PCR) was performed as per manufacturer’s instructions (New England Biolabs), with forward and reverse primer pairs (5′-3′) L-1:

- TLR4: ATG GCA TGG CTT ACA CCA CC
- CD86: TGT TTC CGT GGA GAC GCA AG and TTG AGC CTG TGT AAA TGG GCA;
- CD206: CTC TGT TCA GCT ATT GGA CGC and CGG AAT TTC TGG GAT TCA GCT TC;
- TLR4: ATG GCA TGG CTT ACA CCA CC and GAG GCC AAT TTT GTC TCC ACA;
- FAK(PTK2): TTA GGC GAT CCT ATT GGG AGA TG and TTC TTA GTG TTT GGG CCT TGA CA;
- Src: GAA CCC GAG AGG GAC CTT C and GAG GCA GTA GGC ACC TTT TGT;
- Argnase-1: CAG AAG AAT GGA AGA GTC AG and CAG ATA TGC AGG GAG TCA CC;
- GAPDH: TGC ACC ACC AAC TGC TTA G and GGA TGC AGG GAT GAT TGT C.

All samples were assayed in triplicate and normalized to the GAPDH messenger RNA (mRNA) abundance.

2.9 | Western blotting

Whole cell lysates were prepared according to Minute Total Protein Extraction Kit protocol (NCM Biotech, China) and the protein concentration was measured by the BCA Protein Assay Kit (Thermo Scientific Pierce, USA). A standard western blotting procedure was followed using rabbit anti-mouse antibodies for AKT, SRC, FAK, P-FAK(Tyr861), P-FAK(Tyr397), and MyD88 (CST and Affinity). The membranes were incubated with HRP-conjugated anti-rabbit IgG antibodies (Thermo Scientific). Signals were visualized with Chemiluminescent HRP substrate (EMD Millipore) and analyzed by Image Lab 6.0 software.

2.10 | Statistical analysis

All data were analysed using GraphPad Prism 6 software. Plasma FN levels in sepsis patients use one-way ANOVA comparisons adjustment for comparisons of three or more groups. SOFA scores are ranked data, and the inflammatory markers including pFN are continuous measurement data, so the correlation analysis was conducted by Spearman’s rank correlation method. Survival data were analyzed using the log-rank test. For measurements of bacterial CFU, cytokine concentration, cell phagocytosis rate, and FCM data, groups were compared using two-way repeated measures ANOVA. The remaining in vitro data were analyzed by one-way ANOVA. All cytokines concentrations and phagocytosis rates were expressed by median (quartile), except for mouse pFN concentration with mean ± SD which followed normal distribution. A p value < .05 was considered statistically significant for all experiments.

3 | RESULTS

3.1 | Plasma FN can reflect the severity and prognosis of patients with progressive sepsis

From November 26, 2016, to December 20, 2018, we screened more than 300 patients in the intensive care unit at our hospital, which resulted in the enrollment of 48 infection patients without sepsis and 50 patients with sepsis, of which 16 patients with sepsis died in the end (Table 1). For better study, we define advanced sepsis as one that lasts more than 1 week in clinical practice, and early sepsis as one that lasts no more than 1 week after sepsis diagnosis. We collected plasma samples from these 50 sepsis patients in multiple time points, and the concentration values of CRP, IL-6, PCT, and FN of plasma were all non-normally distributed. The plasma FN (pFN) in early sepsis patients [246.5 (147.3, 535.8) µg/ml] and advanced sepsis patients [158.0 (87.6, 259.2) µg/ml] were significantly lower than that of the normal controls [383.7 (322.5, 444.6) µg/ml] (p < .05 and p < .0001). There was no significant difference between patients with early sepsis and those with infections without sepsis. Compared
with advanced sepsis patients, the pFN concentration of death patients [118.9 (81.65, 182.4) µg/ml] shows the lowest value ($p < .01$) (Figure 1). The concentration of pFN was negatively correlated with the SOFA scores in advanced sepsis patients ($r = -.720, p < .001$), specifically, the higher SOFA scores were, the lower the concentration of pFN was. By contrast, the concentration of IL-6, CRP, and PCT was positively correlated with SOFA scores in advanced sepsis patients, the analysis of correlation $r$ value were .390, .367, and .545, $p < .001$, respectively (Table 2). The results suggested that the correlation between pFN and SOFA score was higher than other commonly used inflammatory markers in advanced sepsis patients.

### 3.2 | Fn KO mice showed enhanced lethality and impaired bacterial clearance during sepsis after CLP

To investigate the impact of Fn gene knockout on the mortality of sepsis mice, we had successfully constructed tamoxifen-induced Fn gene conditioned knockout mouse. Within 72 h after establishment of the modified CLP sepsis model, the mortality of WT and FnloxP groups was slightly higher than that of the Fn KO group (no statistical difference), but the mortality in Fn KO group increased significantly after 72 h. Observed 20 days, the total mortality of the Fn KO group finally reached 85.0%, while that of the WT and FnloxP groups was 50.0% and 55.0%, respectively. The total mortality of Fn KO group was significantly higher than other groups ($p < .05$), when exogenous Fn was administered to Fn KO mice 2 h before CLP, survival was improved, and high concentration of FN (20 mg/kg) had a greater effect on prognosis, but there was no statistical difference (Figure 2A).
We performed cardiac puncture blood culture on the three groups of mice after CLP, and mainly detected *E. coli* and *Bacillus subtilis* within 72 h. Six hours after CLP, the median bacterial CFU counts of blood were $7.31 \times 10^3$ in *Fn* LOX group, $0.75 \times 10^4$ in *Fn* LoxP group, and $0.39 \times 10^4$ in WT group, with the value of the *Fn* KO group was significantly increased compared to the other two groups ($p < .001$) (Figure 2B). In the bacterial intraperitoneal injection model, 6 h after *E. coli* and *Enterococcus* intraperitoneal injection, the *E. coli* CFU counts from peritoneal fluid in WT, *Fn* LoxP, and *Fn* KO group were $6.30 \times 10^4$, $7.55 \times 10^4$, and $1.10 \times 10^4$, respectively, indicated that the *Fn* KO group was significantly higher than the other two groups ($p < .01$), and the same result in *Enterococcus* (Figure 2C).

Furthermore, we detected the phagocytic rate of FITC–*E. coli* in mouse peritoneal macrophages (PEM) by FCM. Phagocytic rate median WT versus *Fn* KO at 15 min were $27.2$ ($22.6, 31.1$) % versus $15.9$ ($12.5, 20.6$) %, $p < .05$; at 30 min were $58.2$ ($49.4, 65.3$) % versus $32.0$ ($26.8, 36.5$) %, $p < .01$; at 40 min were $66.6$ ($59.4, 70.2$) % versus $42.3$ ($36.6, 48.3$) %, $p < .05$, respectively. Data show that the macrophage phagocytosis rate from *Fn* KO mice was significantly lower than that from WT mice. What is more interesting is that when WT and *Fn* KO plasma were added to *Fn* KO and WT mice derived macrophages, respectively, the phagocytosis rate of peritoneal macrophages from *Fn* KO mice was significantly increased, while the phagocytosis rate from WT mice decreased (Figure 2D).

In addition, under LPS (500 ng/ml) stimulation, RAW264.7 cells’ phagocytic rates median in the *Fn*0, *Fn*50, and *Fn*100 μg/ml groups were $11.85$ ($9.65, 15.90$) %, $31.15$ ($22.45, 36.50$) %, and $36.95$ ($26.83, 48.75$) % at 30 min, respectively, with the concentration of *Fn* increased, the phagocytic rate increased (Figure 2E).

### 3.3 *Fn* gene knockout affects the cytokine storm in septic mice after CLP

We investigated the degree of inflammation in the abdominal cavity of mice after CLP. Necrosis of the cecum was observed in mice 24 h after CLP, accompanied with suppuration and peritoneal exudation. In addition, congestion of the peritoneum and intestinal wall in the WT group was more obvious than that in the *Fn* KO group (Figure 3A). The concentration of plasma *Fn* began increasing at 18 h after CLP and reached a peak at 48 h in WT ($367.3 \pm 42.3 \mu g/ml$) and *Fn* LoxP ($352.6 \pm 38.4 \mu g/ml$), however, it did not increase in *Fn* KO mice (<20 μg/ml) (Figure 3B).

The concentration of plasma IL-6 increased at 3 h after CLP, reaching a maximum at 12 h. The peak concentration of WT, *Fn* LoxP, and *Fn* KO were 2169 (1464, 2987) pg/ml, 2066 (1415, 3297) pg/ml, and 1541 (1045, 2362) pg/ml, respectively, *Fn* KO mice was lower than that of the other two groups ($p < .05$). Plasma IL-6 decreased gradually after 12 h in three groups, while the IL-6 in *Fn* KO group increased again between 96 and 168 h, the second peak concentration 1981 (312.3, 3630) pg/ml was significantly higher than that of the other two groups (<200 pg/ml, $p < .0001$) (Figure 3D). The changes of plasma TNF-α concentration were similar to IL-6, with second peak concentration 364.8 (193.1, 564.4) pg/ml of *Fn* KO group in 168 h was significantly higher than that of the other two groups (<150 pg/ml, $p < .0001$) (Figure 3C). The levels of plasma IL-10 in *Fn* KO group even showed three peaks, the second peak 490.6 (238.2, 833.6) pg/ml in 48 h and third peak 1928 (246.1, 3029) pg/ml in 168h were significantly higher compared with the other two groups (<200 pg/ml, $p < .01$ and $p < .0001$) (Figure 3E). The plasma G-CSF and CXCL1 reached a peak at 24 h after CLP, and the G-CSF peak concentrations of WT, *Fn* LoxP, and *Fn* KO were 87 378 (73 960, 89 598) pg/ml, 78 962 (65 945, 88 524) pg/ml, and 161 599 (124 070, 193 778) pg/ml, respectively, and CXCL1 peak concentrations were 984.6 (947.9, 1487) pg/ml, 966.2 (856.7, 1604) pg/ml, and 2427 (1444, 8961) pg/ml, respectively. *Fn* KO group were significantly higher than that other two groups ($p < .01$ and $p < .05$) (Figure 3F,G). The concentrations of IL-1β, IL-4, IL-17A, and other factors were not different between the three groups, and many were lower than the detection range.

In the model of inflammation induced by intraperitoneal injection of LPS (10 mg/kg) in mice, the concentrations of IL-6 and IFN-γ in WT (13387 (9836, 16374) pg/ml, 321.2 (287.6, 361.2) pg/ml) and *Fn* LoxP (12487 (9256, 15823) pg/ml, 303.8 (279.1, 351.3) pg/ml) groups were significantly higher than those in the *Fn* KO group (5631 (3740, 8178) pg/ml, 44.4 (23.6, 91.4) pg/ml) at 12 h after LPS injection ($p < .001$ and $p < .0001$) (Figure 3H). And WT and *Fn* LoxP group mice had a higher mortality rate with the increasing dose of LPS (data not shown).

### 3.4 *Fn* gene knockout attenuated monocytes/macrophage polarization in septic mice

In order to determine whether *Fn* gene knockout affects the polarization of monocytes/macrophage in mice, we...
FIGURE 2  Fn KO mice showed enhanced lethality and impaired bacterial clearance during sepsis. (A) Survival of mice during 20 days after CLP (n = 20/group, p < .05), and Fn KO mice were injected with FN (20 or 10 mg/kg, q8h*3days) or same volume of 0.9% NaCl by tail vein (n = 12/group). (B) Bacterial counts in blood 6 h after CLP (n = 8, p < .001). (C) Bacterial counts in peritoneal lavage fluid 6 h after bacterial intraperitoneal injection (n = 8, p < .01). (D) Phagocytosis rate of FITC-labeled E. coli by peritoneal macrophages (PEM) of WT and Fn KO mice over time under different plasma conditions by FCM. (E) Phagocytosis rate of FITC–E. coli by RAW264.7 cells (LPS 500 ng/ml, 30 min stimulus) at different concentrations of FN observed by fluorescence microscope. Data were represented as median, *p < .05, **p < .01, ***p < .001, ****p < .0001
examined the polarization of monocytes/macrophage toward M1 type (CD86 and iNOS) and M2 type (CD206 and ARG-1) in sepsis mice. FCM showing that the expression of CD86 and CD36 on blood monocytes in Fn KO mice was lower than that in WT mice at 12 and 24 h after CLP, and the expression of CD206 on blood monocytes in Fn KO mice was lower than that in WT mice at 24 and 48 h after CLP (Figure 4A). The same results were seen in the peritoneal macrophages after intraperitoneal injection of LPS (10 mg/kg). Quantitative RT-PCR screening suggested that the expression of inducible nitric oxide synthase (iNOS) and arginase-1 (ARG-1) mRNA in peritoneal macrophages of Fn KO group was significantly lower than that of WT group 12 h after LPS injection (10 mg/kg) (p < .05) (Figure 4D).

3.5 | Exogenous FN can promote the polarization of RAW264.7 cells stimulated by endotoxin and affects the secretion of inflammatory cytokines

To further clarify the effect of pFN on the polarization of monocytes/macrophage, macrophage polarization-related molecules were detected in RAW 264.7 cells at 12, 24, and 48 h after stimulation with LPS (1 μg/ml) under different concentrations of FN culture. We found that the expression of CD86, CD36, and CD206 on RAW264.7 cells in FN positive group was significantly higher than that in the FN negative group by FCM (Figure 4C). The mRNA relative expression of CD86, iNOS, CD206, and Arg-1 on RAW264.7 cells was also upregulated significantly in FN positive group at 12–24 h after LPS (1 μg/ml) stimulation.
FIGURE 4  FN can promote monocytes/macrophage polarization and regulate inflammatory cytokine release in inflammation. (A) Expression of CD86, CD36, and CD206 on peripheral blood mononuclear leucocyte in WT and Fn KO mice by FCM over time after CLP. (B) Expression of CD86, CD36, and CD206 on peritoneal macrophages of WT and Fn KO mice by FCM over time injected with LPS (10 mg/kg). (C) Expression of CD86, CD36, and CD206 on RAW264.7 cells stimulated by LPS (1 μg/ml) under different concentrations of FN by FCM. (D) Expression of inducible nitric oxide synthase (iNOS) and arginase-1 (ARG-1) mRNA in peritoneal macrophages of WT and Fn KO group after LPS injection (10 mg/kg). (E) The mRNA relative expression of TLR4 in RAW264.7 cells under different FN concentration group at 12 h after LPS (1 μg/ml) stimulation. (F) The mRNA relative expression of CD86 and iNOS in RAW264.7 cells under different FN concentration group at 12 h after LPS (1 μg/ml) stimulation. (G) The mRNA relative expression of CD206 and Arg-1 in RAW264.7 cells under different FN concentration group at 24 h after LPS (1 μg/ml) stimulation. (H, I) The IL-1β, IL-6, and TNF-α mRNA relative expression in RAW264.7 cells stimulated by LPS under different concentrations of FN for 12 h. (J) The concentration of IL-6 and TNF-α in the supernatant of RAW264.7 cells stimulated by LPS (1 μg/ml) at different concentrations of FN for 12 and 24 h by ELISA. (K) NO concentration in the supernatants of RAW264.7 cells stimulated by LPS (1 μg/ml) for 6, 12, and 24 h by colorimetric method. FCM and ELISA data were represented as median, RT-PCR data were represented as mean ± SD (n = 6 mice/group or time points). ****p < .0001, ***p < .001, **p < .01, *p < .05
(Figure 4F,G). We found compared with the FN negative group, the mRNA relative expression of TLR4 was downregulated in the FN positive group (p < .01) (Figure 4E).

We also measured the expression of IL-1β, IL-6, and TNF-α in RAW264.7 cells or in the supernatant. After 12 h of LPS stimulation, the IL-6 and TNF-α mRNA relative expression of RAW264.7 cells in FN 50 μg/ml group were significantly higher than those in the FN negative and FN 100 μg/ml group, FN 100 μg/ml group was even lower than FN negative group (Figure 4I). However, the levels of IL-6 in the supernatant of FN 50 μg/ml and FN 100 μg/ml groups were significantly higher than those of the FN negative group (p < .01). Until 24 h under LPS stimulation, only FN 50 μg/ml group was significantly higher than the other two groups, and no significant difference was found between the FN 0 and FN 100 μg/ml groups (p < .0001) (Figure 4J). As to TNF-α, there was no significant difference in the supernatant among the three groups after 12 h of LPS stimulation, similar to IL-6, after continuous stimulation for 24 h, the FN 50 μg/ml group was significantly higher than the other two groups, and no significant difference was found between the FN 0 and FN 100 μg/ml groups (p < .01) (Figure 4I). As to IL-1β, the mRNA relative expression in FN 100 μg/ml and FN 50 μg/ml groups was significantly higher than those in the FN negative group (p < .0001) (Figure 4H), but the concentration of IL-1β in the supernatant was under the detection range by ELISA. We also measured the expression nitric oxide (NO) in the supernatant, data showed that the concentration of NO in supernatant was highest in FN 50 μg/ml and lowest in the FN 100 μg/ml group within 12 to 24 h. There were significant differences between the other groups (p < .001), except for FN25 and FN50 μg/ml (Figure 4K).

3.6 FN affect monocytes/macrophages function by activating integrin α5β1/FAK/Src pathway

To explore the mechanisms by which FN affects monocytes/macrophages function, we examined key proteins in the signaling pathways of known FN receptors. We found that the expression of FAK and Src at the mRNA and protein levels in peritoneal macrophages of Fn KO mice was both downregulated compared to those of the WT mice between 12 and 24 h after LPS injection, and Y861 was positive for FAK phosphorylation site (Figure 5A,B).

In RAW264.7 cell experiments, the expression of FAK and Src at the mRNA and protein levels in FN positive group was both higher than that in FN negative group under LPS stimulation, respectively (Figure 5C,D). The expression of FAK was downregulated under intervention with integrin α5β1 receptor antagonist ANT-161 (100 μmol/L), but not under intervention with integrin αβ3/5 inhibitor Cilengitide (10 μmol/L). FAK phosphorylation inhibitor Y15 (50 μmol/L) inhibited FAK autophosphorylation but did not inhibit FAK expression (Figure 5E). We also found that the expression of AKT in FN positive group was higher than that in FN negative group under LPS stimulation, however, the increase in MyD88 expression in FN positive group was not obvious (Figure 5F).

4 DISCUSSION

Sepsis is a severe critical illness syndrome and remains a leading cause of death in intensive care units (ICUs) worldwide. Despite aggressive ICU care, early resuscitation, and source control, mortality remains dismally high between 30% and 80%. Currently, commonly used clinical biomarkers of inflammation include PCT, CRP, IL-6 etc, and these can only be used to assess the degree of inflammation in sepsis, but not to predict the severity and prognosis of sepsis. Besides IL-6, no other inflammatory markers have been found that can be used as therapeutic targets. As early as the 1980s, Eriksen HO et al. reported that serial determinations of pFN are essential in patients under intensive care, Brodin et al. reported that the plasma fibronectin concentration in suspected septicemia is related to severity of sepsis. However, the sensitivity used to diagnose sepsis is not high enough due to the pFN concentration has a large range of variation in different stages of sepsis patients, it has not been converted to clinical use. In this study, we confirmed again a significant reduction of pFN in sepsis patients. Our study further revealed that pFN fluctuates significantly in patients with early sepsis, even many patients’ pFN levels are above baseline. However, pFN was significantly reduced in patients with advanced sepsis. Considering that pFN is mainly produced by hepatocytes, we also noted that deterioration of liver function in advanced sepsis patients, which may be the cause of the low pFN. So far, it has not been determined whether the decrease in pFN in sepsis patients is due to decreased synthesis or increased consumption, due to the difficulty of obtaining clinical liver specimens. Here, we analyzed the correlation of the levels of commonly used inflammatory markers and the SOFA scores, different from PCT, CRP, and IL-6, pFN level was negatively correlated with SOFA score in advanced sepsis, and the correlation coefficients were higher than other inflammatory markers. Furthermore, persistently low pFN levels suggest a poor prognosis in sepsis. The results of this study again suggest that pFN can be used as a prognostic indicator in advanced sepsis.
Cytokine storm (CS) and multiple organ dysfunction syndrome (MODS) are two major causes of death in sepsis patients, therefore, increasing attention has been paid to the treatment of CS in the acute phase and prevention of MODS during the immunosuppressive period. Previous studies have preliminarily confirmed that the survival of patients with sepsis could be improved by adding fibronectin-rich cryoprecipitate, however, the mechanism of anti-sepsis of pFN has not been elucidated due to the limitations of animal models. We constructed a tamoxifen-induced Fn conditional knockout mouse model. It was found that Fn gene knockout did not affect the immune parameter of mice, including blood routine and cell subsets in important immune organs. In this study, we demonstrated that Fn gene knockout increased the total mortality in mice with sepsis induced by CLP for the first time, it was further confirmed that exogenous FN supplementation to Fn KO mice could block the high mortality of sepsis models after CLP. Although there was no statistical difference compared with the saline group and negative control group, which may be related to the insufficient sample size.

On further study we found that 12 h after CLP, the concentration of plasma pro-inflammatory cytokines such as IL-6 and TNF-α was significantly increased in all groups of mice, which began to decline after 48 h. But with the
re-release of pro-inflammatory cytokines 72 h after CLP in Fn KO mice, the mortality rate increased significantly in group of Fn KO mice. For the first time, we found that plasma inflammatory cytokines showed double peaks in Fn KO mice after CLP, which are thought to be related to the loss of the early pFN increase response, but the details of the mechanism have not been spelled out.

However, the reverse survival data were observed in the model of intraperitoneal injection of LPS. Mice in the WT and Fn loxP group died first with the increasing dose of LPS, when the LPS dose reached 25 mg/kg, all mice in WT and Fn loxP group died within 48 h, while in the Fn KO group, the mortality rate was only half that of the control group (data not shown) due to lower expression of plasma inflammatory cytokines. Considering that the model of intraperitoneal injection of LPS merely reflects the early state of sepsis, which is similar to CLP model within 72 h. The results suggested that physiological level of pFN could promote the body’s inflammatory response to endotoxin, FN deletion can inhibit cytokine storm in the early stage of sepsis.

From the anti-infective perspective, the function of FN has not been reported systematically. Previously, Kwon et al. showed that injection of exogenous FN into “partial hepatectomy + LPS injection” rat model could significantly reduce the degree of the inflammatory response. Other studies have reported that FN can inhibit D-GAL+LPS-induced liver failure. Similarly, our previous studies also confirmed that exogenous FN can protect against liver injury of D-GAL+LPS model mice and inhibit inflammation. In this study, pFN was shown to have a bidirectional role in immune regulation of inflammation. First, pFN can promote the release of inflammatory cytokines in the early stage of endotoxemia. However, with the increasing level of plasma FN in the acute phase after CLP, the secretion of inflammatory cytokines in plasma decreased significantly, suggesting that a high concentration of pFN could inhibit the secretion of inflammatory cytokines, which was also confirmed in RAW264.7 cell studies. In addition, the peak concentrations of G-CSF and CXCL1 in plasma of Fn gene KO mice after CLP were significantly higher than those of WT and Fn loxP mice, both of these cytokines induced and had chemotactic effects on neutrophils. It is suggested that the feedback of neutrophil induction in Fn KO mice under sepsis is significantly enhanced, and the exact mechanism is unclear.

Immunomodulatory disorder is the main cause of death in sepsis patients. Monocyte/macrophages are important component of the innate immune system, they are the first line of defence for the pathogens. In the inflammatory conditions, macrophages can polarize in M1-type (classic active type), which is involved in phagocytosis, killing pathogens, and secreting pro-inflammatory cytokines, and activates adaptive immune responses. As the inflammation progresses, M2-type (alternative activation type) gradually emerged, which is involved in anti-inflammatory and damaged tissue repair. INOS and ARG-1 are classical markers of M1 and M2 macrophages, respectively. The expression of M1 and M2 related molecule in Fn KO mice after CLP or LPS injection was lower or delayed compared to the WT group, suggests that the absence of FN inhibits the polarization of monocytes/macrophages, thereby affecting the related functions of macrophages.

We also found that the ability to remove bacteria was weakened after FN deletion in mice. This demonstrated that FN has the ability to promote phagocytosis and kill bacteria in mouse. Partly because FN molecules have binding sites for bacterial surface adhesion protein (FN-binding protein, FnBPs), which is an important adhesion molecule mediates FN adhesion and clearance. Second, CD36 is an important component of the scavenger receptor on macrophages, whose upregulation by FN explains the ability of FN to promote macrophage phagocytosis. Another reason may be that Fn knockout affects the capture of bacteria by neutrophils in vivo, pFN can promote thrombosis and enhances blood coagulation. The local clot can further provide an environment for the generation of neutrophil extracellular traps (NETs), which further limit bacterial dissemination.

It has been reported that chemerin induces mouse peritoneal macrophages to adhere to the extracellular matrix (FN) through integrin VLA-5 (α5β1) receptor under inflammatory conditions, with the PI3K, AKT and p38 pathways are activated in macrophages. Our studies showed that the expression of AKT, FAK, and Src kinases was decreased in the absence of FN in vivo and vitro, however, there was no difference between the two groups regarding PI3K. Furthermore, phosphorylation of FAK kinase Y861 tyrosine residues was found in inflammatory state. Focal adhesion kinase (FAK) is a tyrosine kinase that is an important member of integrin-mediated signaling and plays an important role in maintaining cytoskeletal stability and cell migration. At present, the prevailing view is that macrophage activation and movement are mediated by PAMPS–TLRs–iNOS–Src–FAK pathways. However, it has also been reported that macrophage movement requires direct activation of cytokines, such as FN or CSF, through α5β1/FAK and αβ1/paxillin signal. In our study, the integrin receptor α5β1 antagonist (ANT-161) inhibited the upregulation of FAK induced by FN, the results suggest that macrophages are activated by signaling of FN-Integrin (α5β1)-FAK/Src in addition to the prevailing view.
However, the exact mechanism of FN in macrophage polarization requires further investigation. It has also been reported that fibronectin polymer activates M1 and M2 macrophages other than co-stimulation by pFN.\(^{60}\) Second, it was found that ANT-161 antagonists failed to inhibit the role of FN in promoting the phagocytosis of RAW264.7 cells, suggesting that other regulatory mechanisms may be involved in the phagocytosis of macrophages mediated by pFN. In addition, we also found that Fn gene knockout affected the number of NK cells in the peripheral blood of early sepsis mice and reducing the production of immunoglobulin in mice after CLP (data not shown), but the detailed mechanism needs further clarification. Together, these findings suggest that FN plays a critical role in regulating the innate immune/systemic inflammatory response in a mouse model of septic shock. It provides a new idea for the control of cytokine storm in sepsis.

There were also shortcomings in our studies, the UBC-cre/ERT2 mice we used are widely expressed in the body, resulting in the systemic knockout of Fn gene. Therefore, the explanation for some of the results was uncertain whether the plasma FN loss or the cellular FN loss is responsible. Tissue specific Fn gene knockout mouse need to be constructed for further study, and further research is needed to confirm our mouse results in human studies.

In conclusion, pFN plays an important role in balancing coagulation and sepsis, it can more accurately reflect the severity and prognosis of patients with advanced sepsis. The absence of FN altered the systemic cytokine release, affected phagocytic function of macrophages and increased the mortality of sepsis mice, suggesting that FN could be a potential therapeutic target in sepsis.

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DISCLOSURES
All authors have read the journal’s policy on disclosure of potential conflict of interest and have none to declare.

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE
The clinical specimen study was approved by the Ethical Commission of the Fujian Medical University Union Hospital and written informed consent was obtained from all subjects. Laboratory mouse were handled in strict accordance with good animal practice as defined by the National Regulations for the Administration of Experimental Animals of China. Animal protocols and experimental procedures were also approved by the Institutional Animal Care and Use Ethics Committee of the Fujian Medical University (Ethical Approval Number: 2017-0135).

MENTION OF ANY MEETING(S)
PRESENTATION
A part of the experimental results has been presented on the 17th International Congress of Immunology (10/2019) in Beijing, China, Poster P1518.

AUTHOR CONTRIBUTIONS
Xiaohong Yuan and Yong Wu designed and performed animal experiments, analyzed and interpreted the data, and drafted the paper. Jia Lin and Qinyong Weng completed the collection and analysis of clinical patient specimens. Xiaohong Yang and Xiaolan Lian supported the animal experiments. Linqing Wu, Xin Li, Ming Tan, and Zhenxing Lin participated in performing laboratory work. Yuanzhong Chen is the project leader, designed experiments, participating in drafting the article, and approved the final version of the article.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available in the methods and results, for additional data are available on request from the corresponding author. Clinical data are not publicly available due to privacy or ethical restrictions.

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