Original Article
C-terminal heparin-binding domain polypeptide derived from plasma fibronectin, rhFNHC36, protects endotoxemia mice by preventing inflammatory responses and increasing the activity of Th lymphocytes

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Abstract: Plasma fibronectin (pFN) could prevent hepatic failure, but it is difficult to obtain sufficient FN from plasma for treatment. So, the study evaluated the therapeutic effects of rhFNHC-36, a recombinant C-terminal heparin-binding domain polypeptide of fibronectin and could be easily obtained from yeast expression system, on endotoxemia mice sensitized by D-galactosamine (D-GalN). After treatment of rhFNHC-36, the 72-hour survival rates were significantly increased, less necrosis occurred in liver and spleen, and increased plasma proinflammatory MCP-1, IL-6 and TNF were attenuated in endotoxemia. In addition, the percentages of Th1 and Th17 cells were higher whereas the Th2 cells were lower. The expression of PD-1 in CD4$^+$ T cells was decreased accompanied by increased expression of CD28. The proliferation, IFN-γ production and proportion of CD44$^+$ CD62L$^-$ cells were restored in CD4$^+$ splenocytes stimulated with anti-CD3/CD28 Abs, and IL-4 was inhibited. M1 markers were down-regulated and M2 markers were up-regulated in LPS-induced peritoneal macrophages treated by rhFNHC-36. These results suggested the therapeutic effects of rhFNHC-36 on endotoxemia mice were achieved by preventing inflammatory response on macrophages polarization and promoting the cellular activity of Th lymphocytes. Importantly, its functions are comparable to pFN and so rhFNHC-36 has the potential to be used clinically.

Keywords: Fibronectin, sepsis, T lymphocytes, macrophage, cytokine

Introduction

Sepsis is characterized by an intense systemic response to infection. The prognosis of sepsis is extremely poor and the mortality is high, with no effective therapy available. Our previous studies indicate that plasma fibronectin (pFN) could prevent hepatic failure induced by endotoxin in mice [1-3]. However, it is almost impossible to obtain sufficient FN from plasma due to its high expense and the risk of blood-infection disease. Therefore, genetic technology has been employed to produce FN. FN contains several domains that serve as scaffoldings for cell anchorage, known as the heparin-binding domain, collagen-binding domain, fibrin-binding domain and cell-binding domain. These domains bind integrin receptors on a variety of cells including fibroblasts, phagocytes, lympho-

cytes and bacteria. In vitro studies indicate that FN modulates a diverse array of cell functions including adhesion, migration, differentiation, apoptosis, morphology change, hematopoiesis and regeneration [4]. Therefore, a polypeptide derived from FN functional domains is a more feasible method for sepsis treatment. In our previous study, purified recombinant C-terminal heparin-binding polypeptide of FN (rhFNHC36) contains three type III homologous structures and their characteristics are tested by heparin-binding activity assay [5]. However, its therapeutic effects on sepsis are still unclear.

Immune dysfunction plays a critical role in the development of sepsis. Immune response in sepsis could be characterized by a cytokine-mediated hyper-inflammatory responses and a subsequent immune-suppressive conse-

quence [2]. Inflammatory responses are modulated during sepsis, and different components of inflammation may be either up-regulated or down-regulated according to the type of cells and their functions. And immune dysfunctions in macrophages and T cells following sepsis are detected. These alterations have been associated with increased morbidity and mortality in septic patients [6]. The initial phase of sepsis is thought, in large part, to be a result of a "cytokine storm" caused by the activation of innate and adaptive immune cells and the systemic release of a large amount of pro-inflammatory mediators, which are produced mainly by monocytes and macrophages. A reduction in lymphocyte population has been observed during sepsis, with a shift in the balance from Th1 to Th2 cytokines, which may play roles in sepsis [7, 8].

Previous studies have shown that pFN or recombinant fibronectin polypeptide antagonizes hepatic failure induced by endotoxin in mice, but the underlying mechanism is unclear [9]. To illustrate the therapeutic functions of recombinant fibronectin polypeptides, rhFNHC36 was selected. It contains multiple binding sites, including those for α4β1, TLR4 and syndecan, and is expressed on macrophage and T lymphocyte [10]. In the present study, we investigated its effects on septic mice, on the polarization of macrophages and on the differentiation of Th cells.

Materials and methods

Purification of pFN and rhFNHC-36 polypeptide

Fibronectin was purified from plasma by gelatin-sepharose chromatography. Human plasma was batch incubated with gelatin-sepharose 4B (Pharmacia, Switzerland). The slurry was poured into a column and washed extensively with Tris-benzamidine buffer (TB buffer, 5 mM benzamidine, 0.05 M Tis-HC1, pH 7.5) followed by 6 M urea. FN was eluted with 6 M urea and dialyzed against PBS, and protein concentrations were determined by optical density at 280 nm. rhFNHC36 polypeptide was produced as described previously [9].

Effect of pFN and rhFNHC-36 polypeptide on endotoxemia mice sensitized by D-galactosamine (D-GalN)

The model was performed according to the method described previously [11], with minor modifications. Briefly, 8-10-week-old specific pathogen-free (SPF) female BALB/c mice were purchased from the Chinese Academy of Sciences (Shanghai). Endotoxemia mice was induced via intraperitoneal (i.p.) injection of 500 μL PBS containing 50 μg/kg LPS (lipopolysaccharide B, E coli 055:B5, Sigma) and 450 mg/kg D-GalN (Sigma). The mice were injected three times with pFN or rhFNHC36 polypeptide (40 mg/kg) at 30 min prior, and 1 hour, 2 hours after LPS and D-GalN treatment. Control mice were injected three times with PBS. Endotoxemia mice were either sacrificed 6 h later for tissue and cell harvest or checked for survival for 72 hours.

Histological experiments

The liver, spleen and lung were fixed in 10% formalin, and embedded in paraffin, whose sections were stained with hematoxylin eosin (HE) and checked under microscope.

Enzyme linked immunosorbent assay (ELISA)

Plasma cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNFα, IL-12p70) were determined by cytometric bead array technique (BD™Cytometric Bead Array Mouse Inflammation Kit, BD Biosciences, San Diego, USA) by FCM on BD FACSVerse (BD Biosciences, San Diego, USA). And the data were analyzed on FCAP Array™ Software (Version 3.0.1) according to the manufacturer's instruction. Plasma FN was detected by ELISA (Fibronectin Mouse ELISA kit, Abcam) according to the manufacturer's instructions.

Isolation and incubation of murine peritoneal macrophages

Peritoneal MØ were isolated as described [12]. In brief, the peritoneal cavity was lavaged with 5 mL ice cold PBS. The resulting cells were washed and resuspended in RPMI 1640 (Hyclone) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/ml streptomycin at a density of 2×10^6 cells/mL. Cells were incubated at 37°C and 5% CO_2 for 2 hours and any non-adherent cells were removed. The remaining adherent cells were stimulated with LPS (10 μg/mL) plus pFN or rhFNHC-36 for 4 and 16 hours, then cells were collected and prepared for subsequent Real-Time PCR (RT-PCR).

RNA isolation and real-time PCR

Total RNA from peritoneal macrophage was extracted with TRIzol reagent (Invitrogen, Life

Technologies), then reverse transcribed with First Strand cDNA Synthesis kit (Fermentas, Life Science). cDNA was analyzed by RT-PCR with SYBR Green Master Mix (Roche). Specific gene expression was normalized to murine β-actin. Relative expression was calculated with $2^{-\Delta\Delta CT}$ method (ABI Prism 7500, Applied Biosystems).

Phenotypic analysis of T lymphocytes

Splenocytes from whole spleen were disaggregated and stained with a panel of T cell specific antibodies, including anti-CD3 APC, -CD4 FITC, -CD25 PE (eBiosciences, San Diego, CA, USA), anti-CD44 PE, -CD62L APC, -CD69 APC, -PD-1 APC, -CD28 PE (Biolegend, USA). Flowcytometric analysis (50,000 events per sample) was performed on FACVerse (BD Biosciences, San Jose, CA) as described previously.

Intracellular staining for TNF-α, IFN-γ, IL-17A and IL-4

Splenocytes were stimulated with 2 μL of Leukocyte Activation Cocktail, with BD GolgiPlug (BD Biosciences, San Jose, CA) for every 1 mL of cell culture (-10^6 cells/mL) and mix thoroughly. Cells were incubated in a 37°C humidified CO_2 incubator for 11 hours. Following activation, the cells were harvested and washed with FACS Staining Buffer, and stained with CD3-APC and CD4-FITC for identifying T helper lymphocyte population (CD3⁺CD4⁺). After washing with FACS buffer, the samples were fixed and permeabilized by adding 250 μL Fixation/Permeabilization solution (BD Biosciences, San Diego, USA) in dark for 20 minutes. After centrifugation, the supernatant was discarded, and the cells were intracellularly stained to detect TNF-α, IFN-γ, IL-17A and IL-4, using anti-IL-17A PE, -TNF-α PE, -IFN-γ PE and -IL-4 PE (eBiosciences, San Diego, CA, USA) for 30 minutes in dark. Finally, the samples were washed with FACS buffer and resuspended in fixation buffer (PBS supplemented with 1% paraformaldehyde). In total, 50,000 events were acquired. CD4⁺ T cells were gated as CD3⁺CD4⁺, and the percentages of cells producing TNF-α, IFN-γ, IL-17A and IL-4 were determined using thresholds based on isotype controls.

CD4⁺ splenic T cell enrichment

Splenic CD4⁺ T cells were enriched by negative selection with more than 88% purity (CD4⁺ T

Cell Isolation Kit II mouse # 130-095-248, Miltenyi Biotec). Briefly, splenocytes were pelleted (300 g for 10 minutes at 4°C) and resuspended in running buffer with 4:1 antibody solution (50 μL per 10^7 cells) at 4°C for 15 minutes. The antibody cocktail contained biotinylated antibodies that recognized CD8a (cytolytic T cells), CD19, CD45R (B cells), CD49b (NK cells), CD11c, CD105, Anti-MHC class II, CD11b (myeloid cells), and Ter-119 (erythrocytes). After antibody binding, the cell suspensions were diluted with 3:2 running buffer: magnetic beads (50 μL per 10^7 cells) at 4°C for 20 minutes. After incubation, the cell suspensions were diluted to 20 × volume with running buffer, pelleted (300 g, 4°C, 10 minutes) and resuspended in running buffer (500 μL). CD4⁺ T cells were collected from flow-through of a magnetic column and washed with 3 × 3 mL running buffer.

Stimulated cytokine analysis

CD4⁺ T cells (1 × 10^6 cells) were enriched from spleen and stimulated with plate-bound mouse anti-CD3 (2 μg/mL, clone145-2C11, eBiosciences) and anti-CD28 (1 μg/mL, clone 37.51, eBiosciences) with or without pFN or rhFN-HC-36 for 72 hours in complete RPMI medium supplemented with 2 mM l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μM β-mercaptoethanol, and 10% FCS. The supernatant cytokines were determined with ELISA kits for IFN-γ, IL-4 (Millipore), following the manufacturers' instructions.

CD4⁺ T cells proliferation by CFSE labeling

The proliferative capacity of CD4⁺ T cells was confirmed by carboxy fluoroscein succinimidyl ester (CFSE) labeling. In brief, cells were extensively washed and resuspended at a final concentration of 1 × 10^7 cells/ml in PBS with 0.1% BSA. CFSE was added at a final concentration of 10 mM and incubated for 15 min at 37°C. Staining was quenched by addition of 5 volumes of ice-cold culture medium and washed with RPMI medium containing 10% heat-inactivated fetal calf serum. Cells then were stimulated with anti-CD3/CD28 in the presence or absence of pFN or rhFNHC-36 for 72 hours, and analyzed on a FACVerse (BD Biosciences, San Jose, CA).

Presentation of data and statistical analysis

The survival studies were analyzed by log-rank test on Prism 4.0 (GraphPad, San Diego, CA).
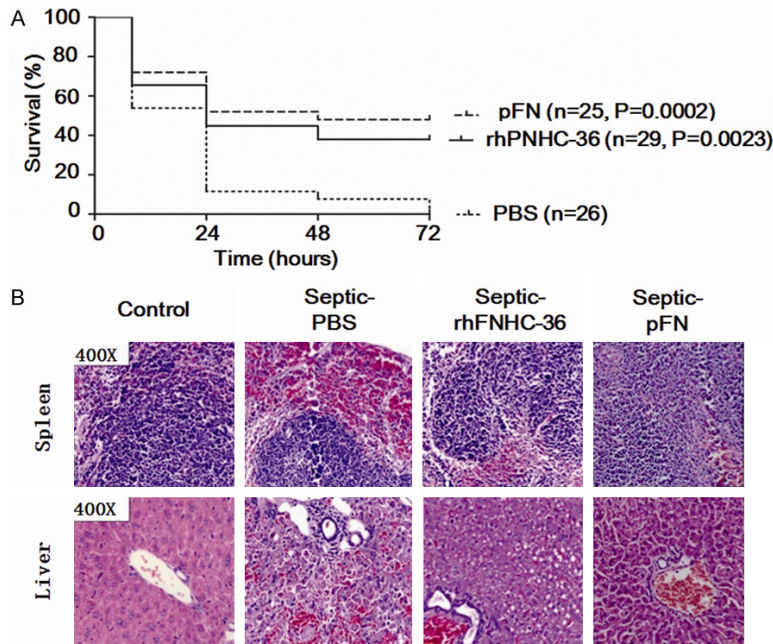
Figure 1. The beneficial effects of pFN and rhFNHC-36 on septic mice. (A) pFN and rhFNHC-36 decreased mortality of LPS/D-GalN-mediated endotoxemia mice. (B) Representative pictures of spleen (A) and liver (B) section with H&E staining.

The data were presented as mean ± SEM for each group. Differences in data were determined with One-way ANOVA (analysis of variance) followed by Fisher's Least Significant Difference post hoc test or Tamhane's T2 test (if equal variance is not assumed) was used for comparison of data from different groups (SPSS 16.0).

Results

rhFNHC-36 or pFN treatment ameliorated endotoxemia

To evaluate possible benefits of rhFNHC-36 and pFN on the lethality of endotoxemia, survival rates were compared. As shown in **Figure 1**, rhFNHC-36 treating group had a survival rates of 52% and 48%, and pFN group had 45% and 38% after 24 hours and 72 hours treatment, respectively, comparing to the control group with 11% and 0% (**Figure 1A**). Therefore, the finding suggested protective effects of rhF-NHC-36 and pFN on endotoxemia and their effects had no difference.

Histopathological studies of liver, spleen and lung were performed to confirm the beneficial effects of rhFNHC-36 and pFN and to investigate events after LPS/D-GalN injection. In accordance with our prior description, massive necrosis was scattered in septic mice liver. Not surprisingly, rhFNHC-36 and pFN treatment reduced the necrosis (**Figure 1B**). Loss of follicular architecture was found in spleens from septic animals, which indicated dysfunction of lymphocytes, while rhFN-HC-36 and pFN treatment ameliorated the changes (**Figure 1B**). For lung, there was no significant difference between septic mice and normal control. These findings suggested the beneficial effects of pFN and rhFN-HC-36 on septic mice.
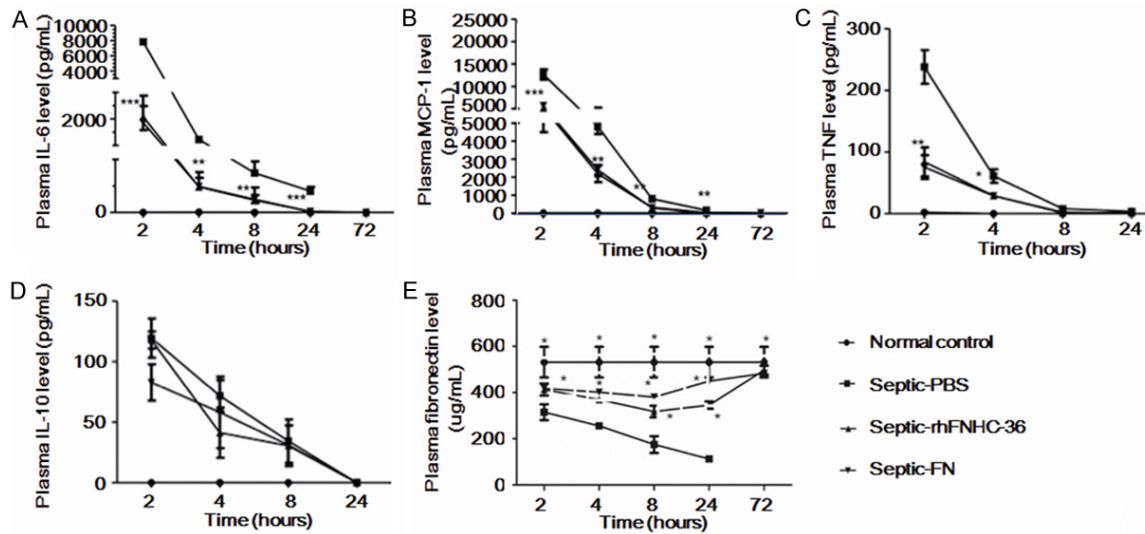
rhFNHC-36 and pFN treatment inhibited the systemic inflammatory responses in mice with endotoxemia

The protective effects of rhFNHC-36 and pFN were associated with the decline of proinflammatory cytokines. Plasma IL-6, MCP-1, TNF and IL-10 were significantly increased in endotoxemia mice comparing to control (**Figure 2A-D**; **Tables 1** and **2**), while plasma IL-4, IL-12 p70 and IFN-γ had no difference (data not shown). IL-6, MCP-1 and TNF were decreased significantly after rhFNHC-36 and pFN treatment, while IL-10 was not attenuated (**Figure 2D**; **Tables 1** and **2**).

Plasma FN was significant decreased continuously in endotoxemia mice during first 2 hours after LPS/D-GalN challenge. rhFNHC-36 and pFN treatment ameliorated the decline of pFN and normal pFN concentration was found at 72 hours (**Figure 2E**). The influence of rhFNHC-36 and pFN on the polarization of peritoneal macrophages was further investigated because macrophages are the major source of the inflammation and their polarization to M1 phenotype are detected in response to bacterial products such as LPS. M1 and M2 markers in LPS induced peritoneal macrophages were

Figure 2. rhFNHC-36 and pFN treatment inhibited the systemic inflammatory responses in mice with endotoxemia. Selected plasma cytokine concentrations in pFN and rhFNHC-36 pretreated endotoxemia mice. The plasma concentrations of 7 cytokines were determined after sepsis. The concentrations of 4 cytokines significantly changed, including (A) IL-6; (B) MCP-1; (C) TNF; (D) IL-10. (E) Plasma FN levels over the course of 72 hours in endotoxemia mice, *$P < 0.05$ compared with Septic-PBS. N = 5 per group.

Table 1. Plasma IL-6

IL-6 (pg/ml)	2 h	4 h	8 h	24 h
Normal control	1.32±0.68***	3.13±1.37***	3.0±3.0***	1.42±1.42***
Septic-PBS	7412±444	1222±78.55	782.7±97.01	398±51.73
Septic-rhFNHC-36	2249±325***,##	496±87.96**,#	305.3±82.63**	29.33±11.39***
Septic-pFN	1981±268***,#	563±101.9**,##	252.0±43.92**	26±11.14***

***$P < 0.0001$, **$P < 0.01$, compared with Septic-PBS; ##$P < 0.01$, #$P < 0.05$, compared with Normal control.

Table 2. Plasma MCP-1

MCP-1 (pg/ml)	2 h	4 h	8 h	24 h
Normal control	8.57±4.88***	8.60±4.887***	6.27±3.58***	9.40±4.97***
Septic-PBS	12649±1126	4815±415.1	786.0±25.53	158.0±34.87
Septic-rhFNHC-36	5184±675.9***,##	2194±472.4**,#	322.0±85.82*	30.67±10.09**
Septic-pFN	5635±499.7***,##	2411±286.0**,##	283.0±116.2**	14.67±8.667**

***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$, compared with Septic-PBS; ##$P < 0.01$, #$P < 0.05$, compared with Normal control.

shown in **Figure 3**, pFN and rhFNHC-36 inhibited the expressions of iNOS, TNF-α, IL-12, IL-1β and IL-6, and increased the expressions of Argnase-1, IL-10 and IL-4. The above results indicated that inflammatory responses in endotoxemia mice were suppressed by rhFNHC-36 and pFN treatment.

rhFNHC-36 and pFN treatment modulated the differentiation of CD4⁺ T cells in endotoxemia mice

The percentages of Th1, Th2 and Th17 subsets were evaluated as well as the expressions of

CD69, CD25, CD28 and PD-1 markers. The percentages of T helper (CD3⁺/CD4⁺) lymphocytes producing TNF-α, IFN-γ and IL-17 were lower in endotoxemia mice than in controls, and the decrease was partly recovered after pFN and rhFNHC-36 treatment (**Figure 4A-C**). The similar result was obtained from T helper lymphocytes producing IL-4 (**Figure 4D**), the increase of which in endotoxemia mice was partly inhibited when treating with pFN and rhFNHC-36. CD25 and CD69 expressions in CD4⁺ T cells were increased in endotoxemia mice comparing with control (**Figure 4E** and **4F**). Meanwhile,
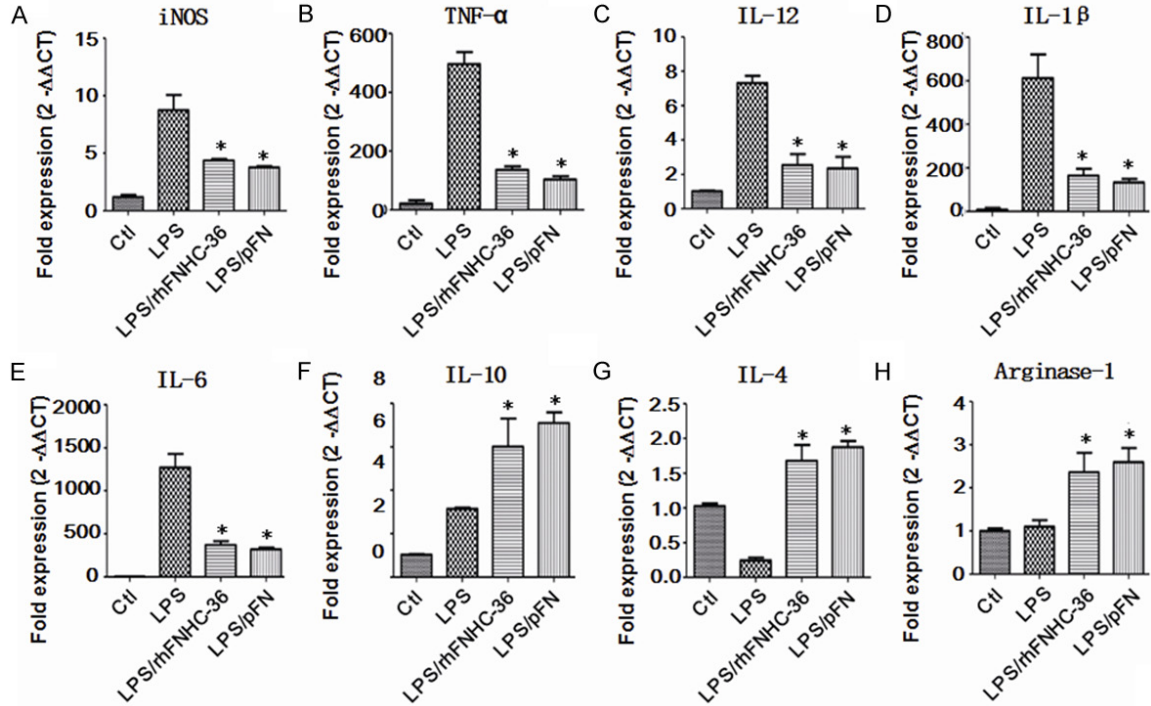
Figure 3. rhFNHC-36 or pFN inhibited M1 polarization in peritoneal macrophages induced by LPS. Peritoneal macrophages from Balb/c mice were cultured with LPS (10 ng/ml) plus rhFNHC-36 or pFN (500 ng/ml) for 4 h. mRNA for (A) iNOS; (B) TNF-α; (C) IL-12; (D) IL-1β; (E) IL-6; (F) IL-10; (G) IL-4 and (H) Argnase-1 was analyzed by RT-PCR. *P < 0.05, comparing to peritoneal macrophages cultured with LPS.
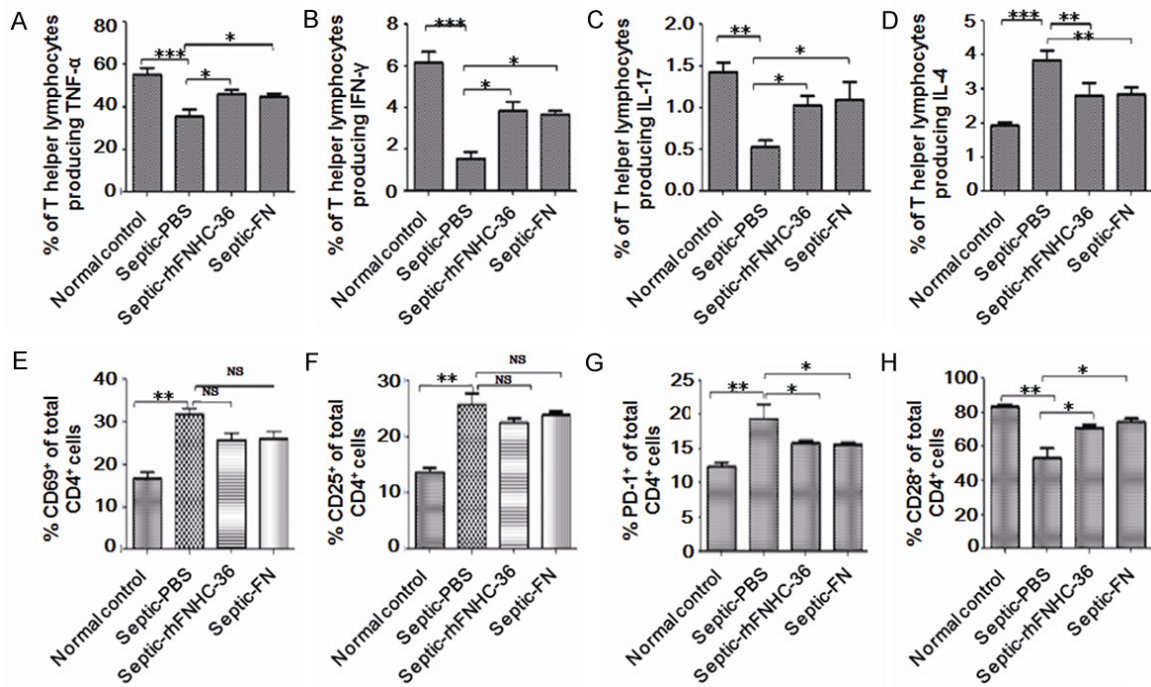
Figure 4. rhFNHC-36 and pFN treatment modulated the differentiation of CD4+ T cells in endotoxemia mice. Percentages of T helper lymphocytes producing TNF-α (A), IFN-γ (B), IL-17 (C) and IL-4 (D). The number of CD69+ (E), CD25+ (F), PD-1+ (G) and CD28+ (H) cells, which were shown as percentages of the CD4+ cell population. Statistically significant differences were indicated by *P < 0.05, **P < 0.01, ***P < 0.001 vs. Septic-PBS; n = 4-5 mice/group.
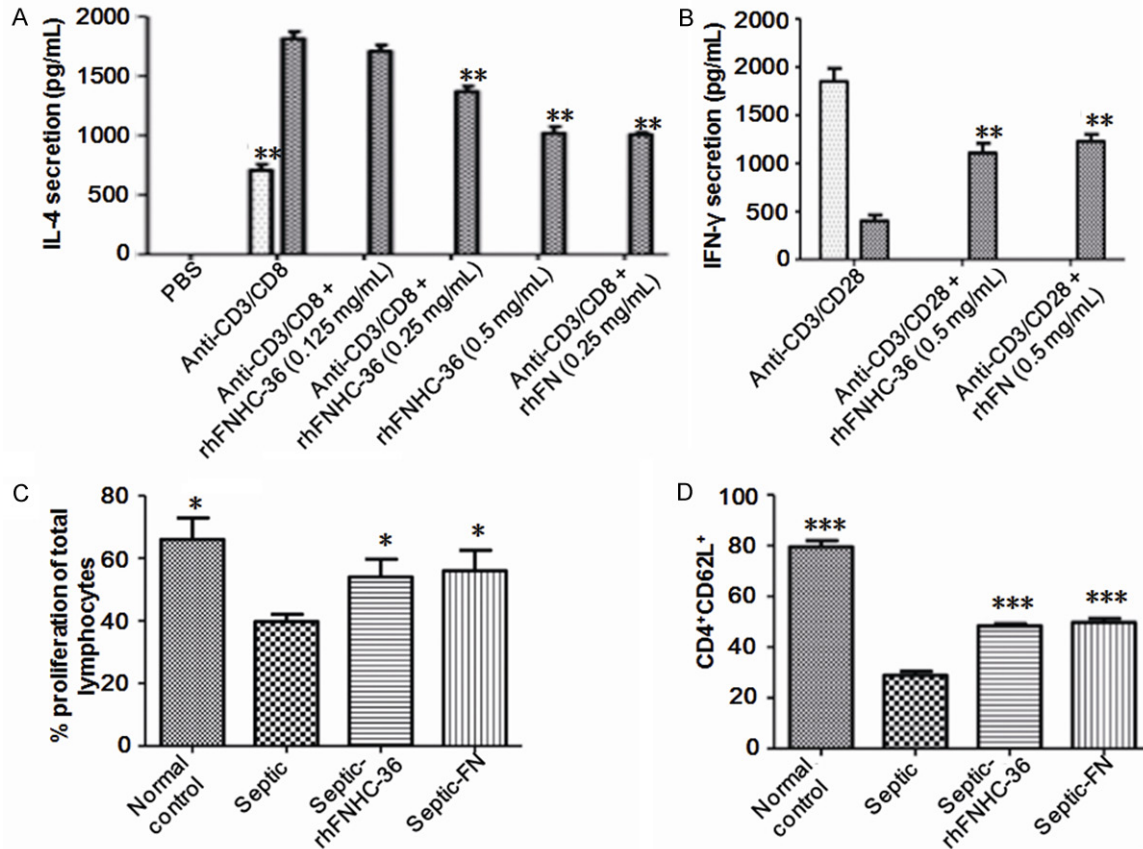
Figure 5. rhFNHC-36 and pFN could regulate the differentiation and functions of CD4+ T cells in endotoxemia mice. Splenic CD4+ T cells were enriched by negative selection and stimulated by anti-CD3/CD28 Abs with or without rhFNHC-36 or pFN for 72 hours, and the levels of (A) IL-4 and (B) IFN-γ were determined by ELISA. n = 5 for each group; *$P < 0.05$, **$P < 0.01$ compared with septic anti-CD3/CD28. CFSE-labeled CD4+ T cells were cultured in the presence of anti-CD3/CD28 with or without rhFNHC-36 and pFN were shown in C, while late activated CD4+ T cells were determined with combination of CD62L and CD44 and shown in D, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. Septic, n = 4-5 mice/group.

positive costimulatory receptors (CD28 in CD4+ T cells) was down-regulated while inhibitory receptors (programmed cell death 1, PD-1) up-regulated (**Figure 4G** and **4H**). However, the expression of PD-1 was inhibited and the expression of CD28 was induced by rhFNHC-36 or pFN treatment, which had no effect on the expressions of CD69 and CD25 (**Figure 4E-H**). These findings illustrated the roles of pFN and rhFNHC-36 in the differentiation of CD4+ T cells in endotoxemia mice.

The cytokine secretion and proliferation of stimulated CD4+ splenocytes in endotoxemia mice

The IFN-γ secretion capacity of CD4+ splenocytes, which was isolated from endotoxemia mice and incubated with anti-CD3/28 Abs, was

diminished (395.6±193.1 pg/mL), whereas that of IL-4 (1814.4±132.82 pg/mL) was evoked comparing with control (1846.4±301.4 pg/mL for IFN-γ, 706.9±115.5 pg/mL for IL-4, respectively) (**Figure 5A** and **5B**). In addition, IFN-γ secretion was restored (1105.7±220.7 pg/mL and 1223.6±190.3 pg/mL, respectively) and IL-4 secretion (1019.7±125.5 pg/mL and 997.42±137.5 pg/mL, respectively) was inhibited by rhFNHC-36 or pFN treatment, respectively (**Figure 5A** and **5B**).

The CFSE (Carboxyfluorescein succinimidyl ester) profiles, which are an effective and popular means to monitor lymphocyte division, revealed a significant decrease of CD4+ T cell proliferation in endotoxemia mice compared with control. Only 39.8±5.06% of precursor CD4+ T cells were undergoing proliferation

when activated by anti-CD3/CD28, compared with 66±15.4% in control. However, increased proportion of CD4+ T cells was detected in the presence of rhFNHC-36 and pFN (56.4±15% and 54±14.3%, respectively) (**Figure 5C**). CD44+/CD62L+ population in CD4+ T cells was significantly decreased compared with control, and this was partly restored by the addition of rhFNHC-36 and pFN (**Figure 5D**). The results suggested that rhFNHC-36 and pFN could regulate the differentiation and functions of CD4+ T cells in endotoxemia mice.

Discussion

Clinical studies indicated that FN levels decreased significantly in severe sepsis patients *in vivo*, and plasma FN levels were associated with the severity of sepsis [13, 14]. When cryoprecipitate with rich FN was injected into sepsis patients, plasma FN would return to normal level [15]. Our previous study is the first to purify FN from fresh frozen plasma, which was then applied to animal model for sepsis therapy. These findings confirmed that FN could reduce the mortality of D-GalN-sensitized mice with decreased cell degeneration, necrosis and apoptosis in mouse liver and liver TNFα, IL-1β and IL-6 mRNA expressions [9]. Although clinical studies and animal experiments suggested the possibility of FN treatment for sepsis, there are still limitations to FN acquisition. FN isolation from human plasma is not inadequate, but there is a risk of transmission of blood-borne infectious diseases. Our study firstly used the *Escherichia coli* expression system expressing the FN cell binding domain (CBD). Animal experiments have shown that rhFN-CBD can reduce mortality in D-GalN-sensitized mice, and improving endotoxemia mouse hepatocyte apoptosis and necrosis [16]. To further understand other FN functional domains, we applied yeast expression system to product both N-terminal and C-terminal of FN polypeptide, rhFNHN-29 and rhFNHC-36 polypeptide, which are heparin-binding domains (HBD) [16]. In this study, preliminary animal experiments confirmed that rhFNHC-36 (amino acid residues 2068-2082, composed of type III 12-14) could play therapeutic roles in sepsis mice and more experiments were applied to discover the mechanism of rhFN-HC-36 functions in sepsis.

Our results confirmed the protective effects of rhFNHC-36 in D-GalN/LPS-induced septic mice

were possibly through the regulation of macrophage polarization, the control of inflammation, the balance adjustment of Th subpopulations, and the improvement of immunosuppressive effects in Th cells. Importantly, its functions were comparable to pFN. Therefore, rhFN-HC-36, which is easily acquired with genetic engineering techniques, could replace pFN and be used to treat the symptoms of sepsis.

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Disclosure of conflict of interest

None.

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