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Notch signaling pathway regulates T cell dysfunction in septic patients

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ABSTRACT

Sepsis disrupts innate and adaptive immune response, and immune disorders may also impact clinical course of sepsis. Notch signaling pathway plays a vital role in T cell modulation and differentiation. The aim of current study was to investigate the immunoregulatory function of Notch signaling pathway to T cells in patients with sepsis and septic shock. Twenty-seven sepsis patients, twenty-five septic shock patients, and twenty-one normal controls (NCs) were enrolled. Notch receptors mRNA levels were semi-quantified by real-time PCR. The absolute numbers of $CD3^+$, $CD4^+$, and $CD8^+$ T cells were measured by flow cytometry. Key transcriptional factors of $CD4^+$ T cells, cytotoxic molecules in $CD8^+$ T cells, and cytotoxicity of $CD8^+$ T cells were investigated. The regulatory activities of Notch signaling inhibition by γ-secretase inhibitor (GSI) on purified $CD4^+$ and $CD8^+$ T cells from sepsis and septic shock patients were also assessed. Notch1 mRNA relative level was significantly elevated in sepsis and septic shock patients when compared with NCs. $CD4^+$ and $CD8^+$ T cells were dysfunctional in sepsis and septic shock, which presented as decreased cell accounts, down-regulation of Th1/Th17 transcriptional factors and cytotoxic molecules (perforin, granzyme B, and FasL), and reduced cytotoxicity of $CD8^+$ T cells. Notch signaling inhibition by GSI increased Th1 and Th17 differentiation of $CD4^+$ T cells. Moreover, GSI stimulation not only promoted perforin, granzyme B, and FasL mRNA expression in $CD8^+$ T cells, but also elevated $CD8^+$ T cell-induced target cell death and IFN-γ/TNF-α production in sepsis and septic shock. The current data suggest that Notch signaling pathway contributes to T cell dysfunction and limited immune response in sepsis.

1. Introduction

Sepsis is a medical emergency which is associated with high mortality. It is estimated that > 30 million peoples are affected by sepsis annually all over the world, resulting in aproximate 6 million deaths every year [1,2]. In most severe form, such as severe sepsis and septic shock, sepsis always causes end-stage organ dysfunction that produce long-term morbidity characterized by severe immune exhaustion and catabolism [2,3]. In the past decades, significant advancements in hemodynamic monitoring, resuscitation measures, and understanding of pathophysiology have significantly improved the outcomes of sepsis [1,2]. There was a decrease trend in mortality in the past two decades in patients with severe sepsis and septic shock [4,5]. However, sepsis remains one the major causes of morbidity and unacceptable mortality in critically ill patients [1]. In 2016, a new definition of sepsis (Sepsis-3) was developed. Sepsis is now defined as infection with organ dysfunction caused by dysregulated host response to infection [6]. Sepsis-induced organ dysfunction and lethality results from the complex interaction between initial inflammatory and later anti-inflammatory responses [7]. Importantly, sepsis disrupts innate and adaptive immunity, which induces immune disorder [8]. Both innate and adaptive immune system dysfunction have been demonstrated to cause impaired ability to eradicate primary infection and lead to occurrence of secondary opportunistic infections [9]. Thus, restoration of immune cell exhaustion, especially $CD4^+$ and $CD8^+$ T cell function recovery, is currently one of the focus for therapeutic approaches to sepsis.

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Notch receptors are transmembrane epidermal growth factor-like repeat-containing proteins, which are pivotal for regulation of cellular proliferation, differentiation, and apoptosis [10]. Notch signaling pathway determines the generation of T cell precursors from hematopoietic stem cells, and biases precursor cells toward different cell linages in a thymus microenvironment [11]. However, controversy remains as to the Notch signaling pathway on regulation of activated T cells. Kondo et al. revealed that Notch signaling pathway mediated the conversion of activated $CD4^+$ and $CD8^+$ T cells into stem cell memory-like T cells by losing expressions of inhibitory molecules, such as programmed death-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 [12]. In contrast, Mathieu et al. showed that Notch signaling inhibition regulated $CD8^+$ T cells activation by induction of PD-1 [13]. Importantly, Notch pathway played a novel and critical role in regulating T cell responses and development [14,15]. Thus, we hypothesized that Notch signaling pathway contributes to T cell dysfunction in the pathogenesis of sepsis. To test this possibility, we examined the Notch receptors expression and status of T cell dysfunction in sepsis and septic shock patients, and accessed the Notch signaling inhibition to $CD4^+$ and $CD8^+$ T cell function *in vitro*.

2. Patients, materials and methods

2.1. Enrolled subjects

The study protocol was approved by the Clinical Research Ethics Committee of both Shaanxi Provincial People's Hospital and First Hospital of Jilin University, and informed consent was obtained from each subject or the guardian. This study examined the 27 patients with sepsis and 25 patients with septic shock who were hospitalized Shaanxi Provincial People's Hospital or First Hospital of Jilin University from March 2018 to December 2018. The diagnosis of sepsis and septic shock was made according to the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) [6]. All enrolled patients were excluded for chronic virus infection and autoimmune disorders. Blood sampling was performed in the early stage of the disease before administration of antibiotics. Twenty-one sex- and age-matched healthy individuals were also enrolled as normal controls (NC). The baseline clinical characteristics of enrolled subjects were shown in Table 1.

Table 1
Baseline clinical characteristics of enrolled subjects.

	NC	Sepsis	Septic shock
Cases	21	27	25
Male/female	15/6	20/7	17/8
Age, years	52.1 ± 5.46	54.00 ± 8.63	50.1 ± 5.99
WBC, $\times 10^9$/L	4.93 ± 1.30	18.15(6.67, 30.26)	19.33(6.75, 31.61)
PCT, μg/L	Not available	2.52(0.88, 11.89)	9.16(1.58, 13.23)
CRP, mg/L	Not available	6.12(3.91, 73.95)	54.91(6.59, 82.97)
ALT, IU/L	22(17, 41)	78(45, 141)	81(58, 120)
sCr, μmol/L	82.38 ± 9.78	91(79, 259)	184(97, 293)
SOFA score	Not available	6.52 ± 3.50	7.60 ± 2.99
APACHE II score	Not available	16.26 ± 6.47	17.32 ± 5.87
28 days death	Not available	10	16
Site of infection			
Lung	Not available	9	8
Abdominal cavity	Not available	12	8
Blood stream	Not available	4	7
Skin and soft tissue	Not available	2	2
Micro-organism			
Gram-positive	Not available	7	9
Gram-negative	Not available	18	15
Fungus	Not available	2	1

NC, normal control; WBC, white blood cells; PCT, procalcitonin; CRP, C reaction protein; ALT, alanine aminotransferase; sCr, serum creatinine; SOFA, sepsis related organ failure assessment; APACHE, acute physiologic and chronic health evaluation.

2.2. Peripheral blood mononuclear cells (PBMCs) and serum preparation

PBMCs were isolated from anti-coagulant peripheral bloods using Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA) by density gradient centrifugation. Serums were isolated from coagulant peripheral bloods by centrifugation at 1000 $\times g$ for 10 min.

2.3. $CD3^+$ T, $CD4^+$ T and $CD8^+$ T cells purification

$CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells were purified using $CD3^+$ Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany), $CD4^+$ Cell Isolation Kit (Miltenyi), and $CD8^+$ Cell Isolation Kit (Miltenyi), respectively. The purity of enriched cells was > 95% by flow cytometry determination.

2.4. Cell culture and stimulation

$CD4^+$ T or $CD8^+$ T cells were seeded into 24-well plates with the concentration of 10^5/mL, and then were cultured in RPMI1640 that was supplemented with 10% of fetal bovine serum (FBS) at 37 °C under 5% CO_2 condition. $CD4^+$ T or $CD8^+$ T cells were stimulated with γ-secretase inhibitor (GSI) LY-411575 (final concentration 1 μmol/L; Adooq Bioscience, Irvine, CA,6 USA) for 24 h. In some experiments, GSI-stimulated $CD8^+$ T cells from HLA-A*0201 restricted patients were co-cultured for 48 h with HCT-116 cells, which was also HLA-A*0201 restricted [16]. Anti-CD3/CD28 (eBioscience, San Diego, CA, USA; final concentration 1 mg/mL) was added for maintenance of $CD8^+$ T cells activity. Cells and supernatants were harvested for further analyses.

2.5. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells using RNeasy Minikit (Qiagen, Hilden, Germany). 1 μg of total RNA was reversely transcribed with random hexamers using PrimeScript RT Master Mix (TaKaRa Biotech, Beijing, China). Real-time PCR was performed using TB Green Premix Ex *Taq* II (TaKaRa Biotech). Relative target gene expression was semi-quantified by $2^{-\Delta\Delta Ct}$ method using ABI 7500 System Sequence Detection Software (Applied Biosystems, Foster, CA, USA). The sequences of primers were used as previously described [17–20].

2.6. Enzyme linked immunosorbent assay (ELISA)

Interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) expression in cultured supernatants was measured using commercial ELISA kits (eBioscience, San Diego, CA, USA).

2.7. Flow cytometry

20 μL of BD Tritest CD4 FITC/CD8 PE/CD3 PerCP Reagent (BD Bioscience, San Jose, CA, USA) was added into BD Trucount absolute count tubes (BD Bioscience). 50 μL of anti-coagulant peripheral bloods were then added for a 15-min incubation in dark. Red blood cells lysis buffer was added for another 15-min incubation. Data were acquired using a FACS Aria II flow cytometer (BD Bioscience), and the absolute $CD3^+$, $CD4^+$, and $CD8^+$ T cell numbers were analyzed using MultiSET Software (BD Bioscience).

Purified $CD4^+$ T cells or $CD8^+$ T cells were stained with anti-human CD3, CD4, CD8, IFN-γ, interleukin-4 (IL-4), FoxP3, IL-17 (eBioscience) for detection of immune cell subsets. Data were acquired using a FACS Aria II flow cytometer (BD Bioscience, San Jose, CA, USA), and were analyzed using FlowJo Software Version 8.6 (Tree Star Inc., Ashland, OR, USA).

2.8. Cytotoxic assay

Target HCT-116 cell death was determined by measurement of

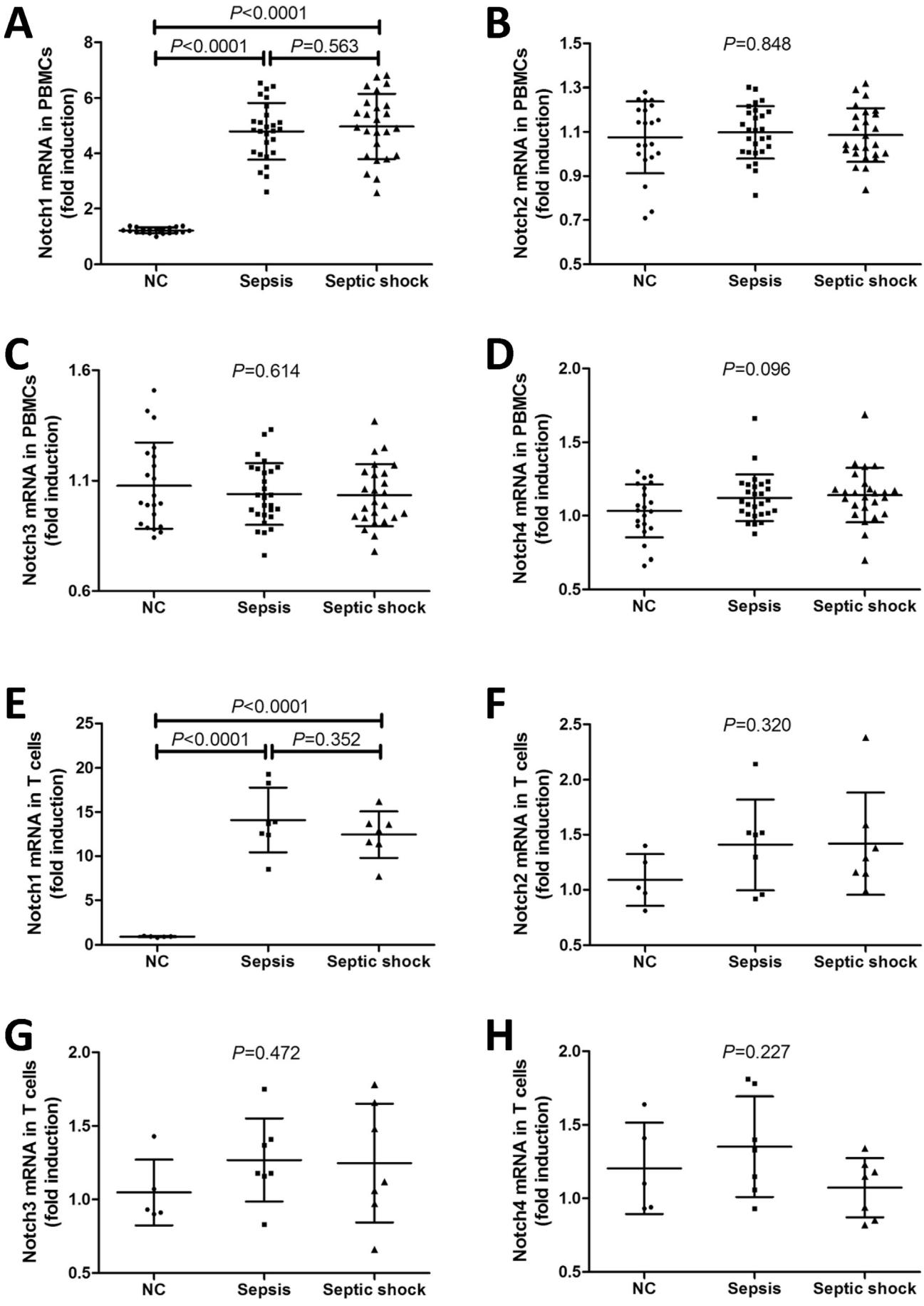

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Fig. 1. Notch receptor mRNA relative levels in normal controls (NC, $n = 21$), sepsis ($n = 27$), and septic shock ($n = 25$). PBMCs were isolated from all enrolled subjects. $CD3^+$ T cells were purified from five NCs, seven septic patients, and seven septic shock patients. Notch mRNA expressions were semi-quantified by real-time PCR. (A) Notch1, (B) Notch2, (C) Notch3, and (D) Notch4 mRNA relative level in PBMCs was compared among NC, sepsis, and septic shock. (E) Notch1, (F) Notch2, (G) Notch3, and (H) Notch4 mRNA relative level in $CD3^+$ T cells was also compared among NC ($n = 5$), sepsis ($n = 7$), and septic shock ($n = 7$). Horizontal bars indicated the mean values, while error bars indicated the SD. The individual level for each subject was shown. Significance was calculated using One-Way ANOVA and SNK-q test.

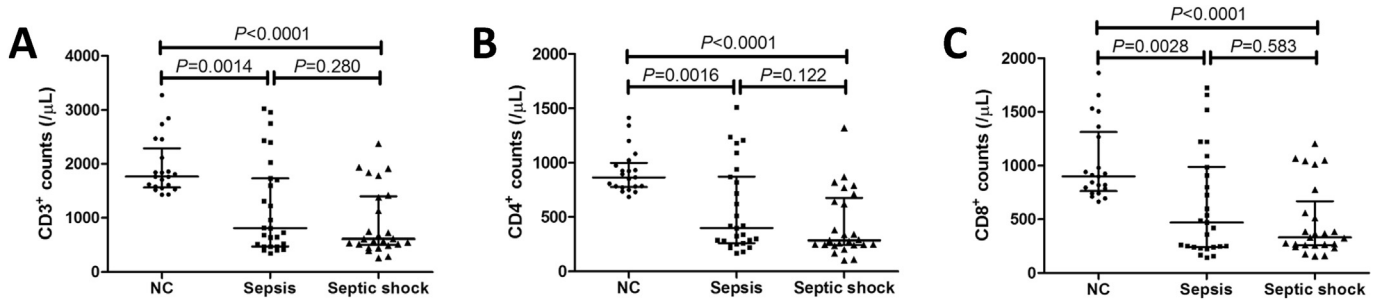

Fig. 2. $CD3^+$, $CD4^+$, and $CD8^+$ T cell counts in normal controls (NC, $n = 21$), sepsis ($n = 27$), and septic shock ($n = 25$). $CD3^+$, $CD4^+$, and $CD8^+$ T cell counts were analyzed by flow cytometry. (A) $CD3^+$, (B) $CD4^+$, and (C) $CD8^+$ T absolute number was compared among NC, sepsis, and septic shock. Horizontal bars indicated the median values, while error bars indicated the interquartile range. The individual level for each subject was shown. Significance was calculated using Kruskal-Wallis test and Dunn's multiple comparison tests.

lactate dehydrogenase (LDH) expression in the cultured supernatants using LDH cytotoxicity kit (Beyotime, Wuhan, Hubei Province, China). The LDH expression in the supernatants of cultured HCT-116 cells was defined as low level LDH control, while the LDH expression in the supernatants of Triton X-100 treated HCT-116 cells was defined as high level LDH control. The percentage of target cell death was calculated using the following formula: (experimental LDH-low level LDH control)/(high level LDH control-low level LDH control) × 100% [21].

2.9. Statistical analysis

All data were analyzed using SPSS version 19.0 for Windows (Chicago, IL, USA). Shapiro-Wilk test was used for normal distribution assay. Variables following normal distribution were presented as mean ± standard deviation, and statistical significance was determined by paired t-test, One-way ANOVA, or SNK-q test. Variables following skewed distribution were presented as median [Q1, Q3], and statistical significance was determined by Kruskal-Wallis test or Dunn's multiple comparison test. Pearson or Spearman correlation analysis was performed for correlation analysis. A P value of < 0.05 was considered to indicate significant differences.

3. Results

3.1. Notch1 mRNA was elevated in PBMCs from patients with sepsis and septic shock

We firstly screen the Notch receptor mRNA expression in PBMCs and $CD3^+$ T cells from patients with sepsis and septic shock. Notch1 mRNA relative level was approximate 5-flod increased in both sepsis and septic shock when compared with NC (all $P < 0.0001$, SNK-q test; Fig. 1A). There was no significant difference of Notch1 mRNA between sepsis and septic shock ($P = 0.563$, SNK-q test; Fig. 1A). However, there were no statistical differences in mRNA levels of Notch2 ($P = 0.848$, One-Way ANOVA; Fig. 1B), Notch3 ($P = 0.614$, One-Way ANOVA; Fig. 1C), or Notch4 ($P = 0.096$, One-Way ANOVA; Fig. 1D) among three groups. There were also no statistical differences of mRNA relative levels corresponding to Notch receptors among patients with Gram-positive, Gram-negative, and fungus infection (all $P > 0.05$, One-Way ANOVA). Furthermore, $CD3^+$ T cells were purified from five NCs, seven septic patients, and seven septic shock patients. Notch receptor mRNA relative levels were also investigated in $CD3^+$ T cells. Notch mRNA expressions in $CD3^+$ T cells presented similar trend as in PBMCs. Only Notch1 mRNA was elevated in $CD3^+$ T cells from sepsis and septic shock (all $P < 0.0001$, SNK-q test; Fig. 1E). There were no remarkable differences of Notch2, Notch3, or Notch4 mRNA among three groups (all $P > 0.05$, SNK-q test; Fig. 1F, G, and H). There were no remarkable correlations between Notch receptors and clinical index (all $P > 0.05$, Pearson or Spearman correlation analysis).

3.2. $CD4^+$ and $CD8^+$ T cells were dysfunctional in patients with sepsis and septic shock

The cell count for $CD3^+$ T cells were significantly down-regulated in sepsis (808[475, 1730]/μL) and septic shock patients (611[504, 1400]/μL) when compared with NC (1764[1564, 2285]/μL) (all $P < 0.01$, Dunn's multiple comparison tests; Fig. 2A). Similarly, The cell counts for $CD4^+$ T cells and $CD8^+$ T cells were also robustly reduced in sepsis (CD4: 399[262, 872]/μL; CD8: 470[244, 989]/μL) and septic shock patients (CD4: 286[244, 676]/μL; CD8: 332[260, 668]/μL) in comparison with NC (CD4: 866[777, 998]/μL; CD8: 900[764, 1315]/μL) (all $P < 0.01$, Dunn's multiple comparison tests; Fig. 2B and C). However, there were no remarkable differences of $CD3^+$, $CD4^+$, or $CD8^+$ T cell counts between sepsis and septic shock patients (all $P > 0.05$, Dunn's multiple comparison tests; Fig. 2).

The mRNA expressions corresponding to key transcriptional factors of $CD4^+$ T cells and cytotoxic molecules of $CD8^+$ T cells in PBMC were semi-quantified by real-time PCR in both PBMCs and $CD3^+$ T cells. Th1 transcriptional factor T-bet mRNA relative level in PBMCs and $CD3^+$ T cells was notably down-regulated in sepsis and septic shock patients when compared with NC (all $P < 0.0001$, SNK-q test; Figs. S1A and 3A). Although there was no significant difference of Th2 transcriptional factor GATA-3 mRNA expression in PBMCs among three groups ($P = 0.111$, One-Way ANOVA; Fig. S1B), GATA-3 mRNA was decreasingly expressed in $CD3^+$ T cells in sepsis and septic shock patients (all $P < 0.01$, SNK-q test; Fig. 3B). Interestingly, regulatory T cells (Tregs) transcriptional factor FoxP3 mRNA was elevated in PBMC from septic shock patients in comparison with those from sepsis and NC (all $P < 0.001$, SNK-q test; Fig. S1C), however, was increasingly expressed in CD3+ T cells in sepsis and septic shock (all $P < 0.0001$, SNK-q test; Fig. 3C). Th17 transcriptional factor retinoid-related orphan nuclear receptor γt (RORγt) was decreased in PBMC and $CD3^+$ T cells from septic shock patients in comparison with those from sepsis and NC (all $P < 0.001$, SNK-q test; Figs. S1D and 3D). Furthermore, mRNA relative

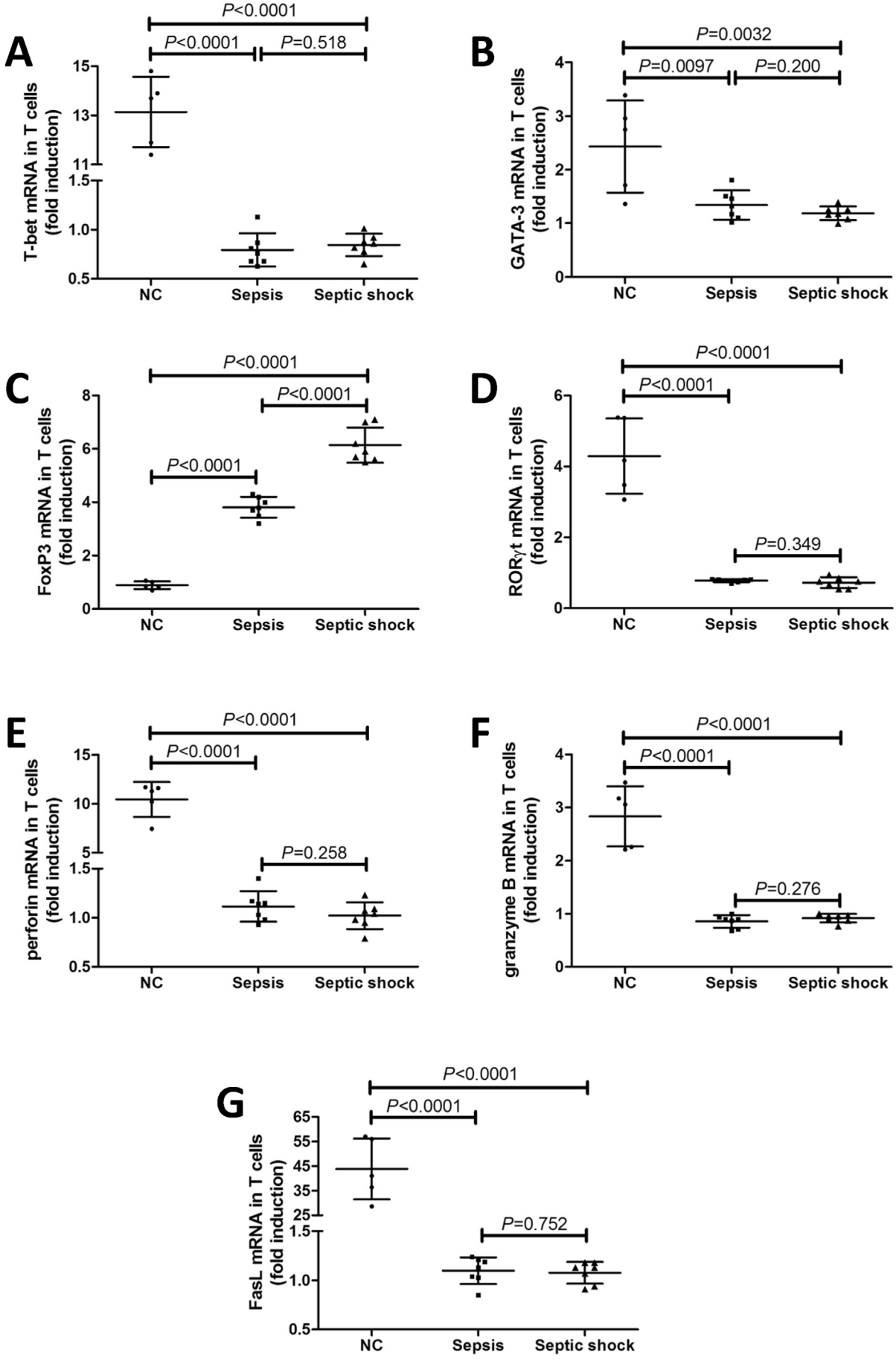

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Fig. 3. mRNA relative levels of key transcriptional factors of $CD4^+$ T cells and cytotoxic molecules of $CD8^+$ T cells in $CD3^+$ T cells from normal controls (NC, $n = 5$), sepsis ($n = 7$), and septic shock ($n = 7$). $CD3^+$ T cells were purified, and mRNA expressions were semi-quantified by real-time PCR. (A) T-bet (Th1 transcriptional factor), (B) GATA-3 (Th2 transcriptional factor), (C) FoxP3 (Tregs transcriptional factor), (D) RORγt (Th17 transcriptional factor), (E) perforin, (F) granzyme B, (G) FasL mRNA relative level in PBMCs was compared among NC, sepsis, and septic shock. Horizontal bars indicated the mean values, while error bars indicated the SD. The individual level for each subject was shown. Significance was calculated using One-Way ANOVA and SNK-*q* test.

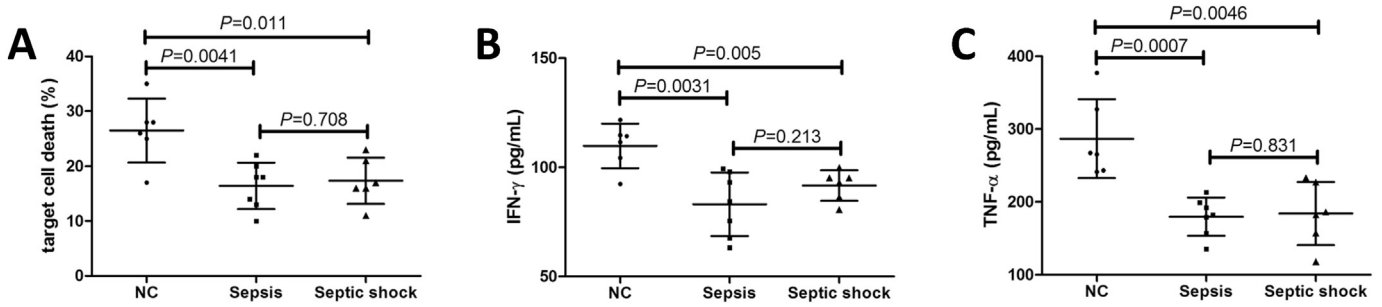

Fig. 4. Target cell death and cytokine production in cocultured system between HLA-A*0201 restricted individuals (NC, $n = 6$; sepsis $n = 7$; septic shock $n = 6$) and HCT-116 cells. 10^5 of purified $CD8^+$ T cells were cocultured with 5×10^5 of HCT-116 cells. Supernatants were harvested 48 h post coculture. (A) Target cell death, (B) IFN-γ expression, and (C) TNF-α expression was compared among NC, sepsis, and septic shock. Horizontal bars indicated the mean values, while error bars indicated the SD. The individual level for each subject was shown. Significance was calculated using One-Way ANOVA and SNK-*q* test.

levels of perforin, granzyme B, and FasL were robustly down-regulated in sepsis and septic shock patients when compared with NC (all $P < 0.0001$, SNK-*q* test; Figs. S1E, F, G, 3E, F, and G).

10^5 of purified $CD8^+$ T cells from HLA-A*0201 restricted individuals (including six NC, seven sepsis, and six septic shock patients) were cocultured with 5×10^5 of HCT-116 cells for 48 h. $CD8^+$ T cells-induced target cell death was down-regulated in sepsis (16.43 ± 4.24%) and septic shock patients (17.33 ± 4.23%) when compared with NC (26.50 ± 5.82%) ($P = 0.0041$ and $P = 0.011$ respectively, SNK-*q* test; Fig. 4A). IFN-γ production in cocultured supernatants were reduced in sepsis (83.01 ± 14.65 pg/mL) and septic shock patients (91.70 ± 7.05 pg/mL) when compared with NC (109.9 ± 10.25 pg/mL) ($P = 0.0031$ and $P = 0.005$ respectively, SNK-*q* test; Fig. 4B). TNF-α production was also decreased in sepsis (179.6 ± 26.30 pg/mL) and septic shock patients (183.8 ± 43.23 pg/mL) when compared with NC (286.7 ± 54.10 pg/mL) ($P = 0.0007$ and $P = 0.0046$ respectively, SNK-*q* test; Fig. 4C).

3.3. Inhibition of Notch signaling promoted Th1 and Th17 effector function within $CD4^+$ T cells from sepsis and septic shock patients

$CD4^+$ T cells were purified from eleven sepsis patients and twelve septic shock patients, and were stimulated with GSI for 24 h. Cells were harvested for flow cytometry analysis. The representative flow dots analysis prior to and post GSI stimulation was shown in Fig. 5A–D. The percentage of Th1 cells ($CD4^+IFN\text{-}\gamma^+$) was significantly elevated in response to Notch signaling inhibition in both sepsis (1.32 ± 0.23% *vs.* 0.92 ± 0.12%, $P = 0.0002$, paired *t*-test; Fig. 5A) and septic shock (1.07 ± 0.15% *vs.* 0.95 ± 0.10%, $P = 0.008$, paired *t*-test; Fig. 5A). However, either Th2 ($CD4^+IL\text{-}4^+$) or Tregs ($CD4^+FoxP3^+$) frequency was comparable between prior to and post GSI stimulation in sepsis and septic shock patients (all $P > 0.05$, paired *t*-tests; Fig. 5B and C). Th17 ($CD4^+IL\text{-}17^+$) percentage was also increased in response to GSI stimulation in both sepsis (15.33 ± 2.42% *vs.* 12.84 ± 2.80%, $P = 0.0067$, paired *t*-test; Fig. 5D) and septic shock (13.28 ± 1.45% *vs.* 11.22 ± 2.20%, $P = 0.0005$, paired *t*-test; Fig. 5D).

3.4. Inhibition of Notch signaling increased $CD8^+$ T cells activity from sepsis and septic shock patients

$CD8^+$ T cells were purified from eleven sepsis patients and twelve septic shock patients, and were stimulated with GSI for 24 h. Cells were harvested for real-time PCR analysis. mRNA relative levels corresponding to perforin (Fig. 6A), granzyme B (Fig. 6B), and FasL (Fig. 6C) were significantly elevated in response to Notch signaling inhibition in both sepsis and septic shock (all $P < 0.05$, paired *t*-tests). Furthermore, 10^5 of stimulated $CD8^+$ T cells from HLA-A*0201 restricted sepsis ($n = 7$) and septic shock ($n = 6$) were cocultured with 5×10^5 of HCT-116 cells for 48 h. $CD8^+$ T cells-induced target cell death was elevated in response to Notch signaling inhibition in sepsis (20.57 ± 3.99%) and septic shock patients (20.17 ± 3.06%) ($P = 0.020$ and $P = 0.010$ respectively, paired *t*-test; Fig. 7A). IFN-γ production in cocultured supernatants were increased in response to Notch signaling inhibition in sepsis (103.7 ± 14.42 pg/mL) and septic shock patients (100.3 ± 5.16 pg/mL) ($P = 0.021$ and $P = 0.0081$ respectively, paired *t*-test; Fig. 7B). TNF-α expression was also increased with GSI stimulation in sepsis (232.9 ± 50.78 pg/mL) and septic shock patients (309.2 ± 76.58 pg/mL) ($P = 0.0036$ and $P = 0.0019$ respectively, paired *t*-test; Fig. 7C).

4. Discussion

In this study, we showed an increased relative level of Notch1 mRNA in peripheral bloods of sepsis and septic shock patients. T cell dysfunction also found in sepsis and septic shock, which mainly presented as down-regulation of cell numbers, decreased Th1/Th17 responses, and reduced cytotoxicity of $CD8^+$ T cells. Moreover, GSI-induced Notch signaling pathway inhibition not only promoted Th1 and Th17 differentiation from $CD4^+$ T cells, but also enhanced direct cytolytic activity and cytokine production of $CD8^+$ T cells from sepsis and septic shock patients. The current data suggested that Notch signaling pathway might contribute to T cell dysfunction/exhaustion in sepsis.

Notch receptors was found to be increasingly expressed in various acute and chronic infections, such as enterovirus 71 infected hand, foot, and mouth disease [22], chronic viral hepatitis [18,20], and *Mycobacterium tuberculosis* infection [23]. Furthermore, Notch signaling molecules, including Notch1, Notch2, and Notch intracellular domain (NICD), were elevated expression in lipopolysaccharide (LPS)-induced sepsis mouse model [24]. LPS stimulation also promoted Notch1 mRNA and protein expression in cultured human umbilical vein endothelia cells *in vitro* [25]. This was partly consistent with our current findings, which revealed the elevated Notch1 expression in the peripheral bloods of sepsis and septic shock. Importantly, Notch signaling pathway was well accepted to be an important regulator of inflammation in sepsis [26]. Suppression of Notch signaling activation reduced LPS-stimulated expression of proinflammatory mediators *in vitro*, such as nitric oxide,

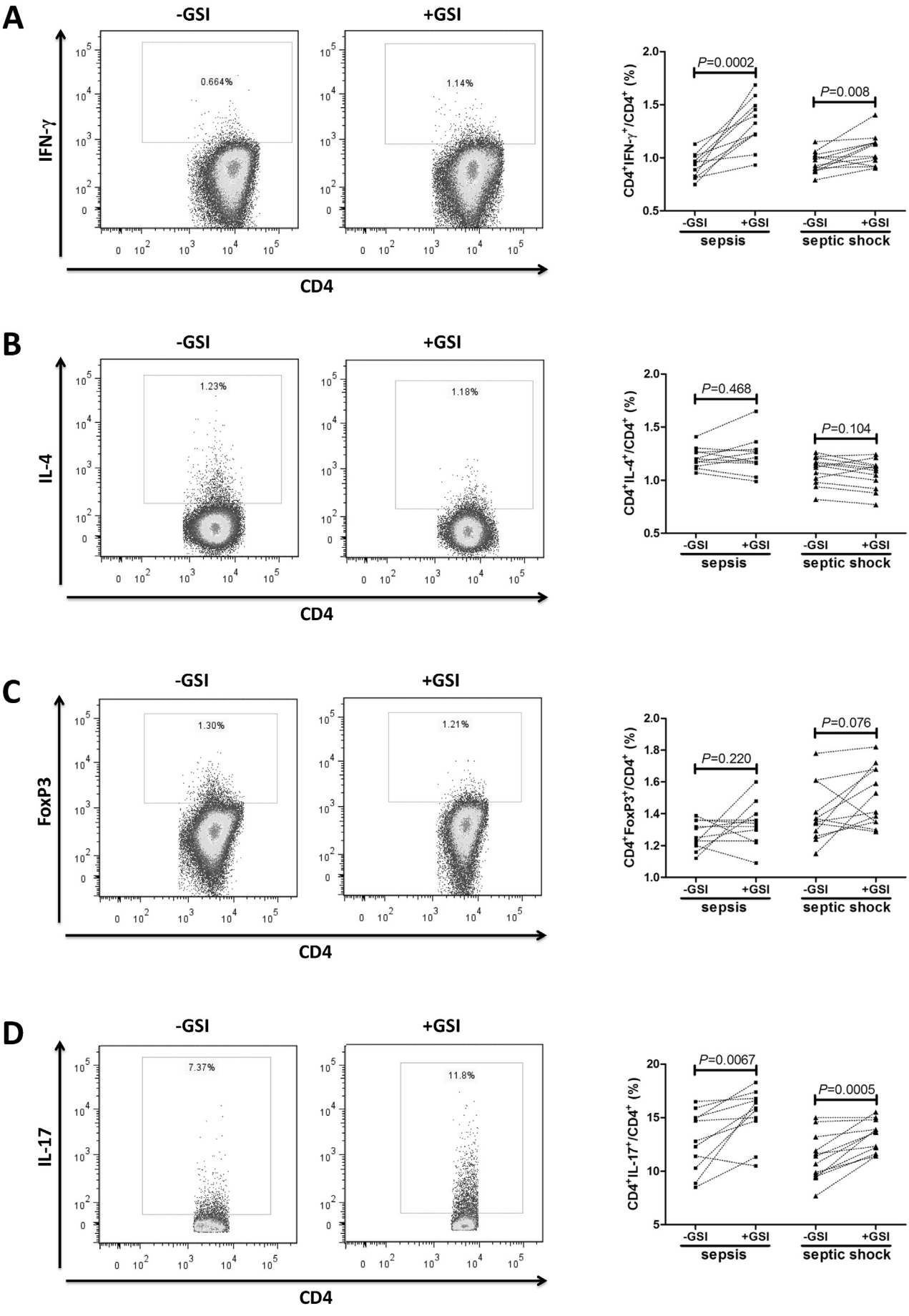

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Fig. 5. CD4$^+$ T cells effector function in response to Notch signaling inhibition in sepsis (n = 11) and septic shock (n = 12). Purified CD4$^+$ T cells were stimulated with GSI for 24 h. Cells were harvested for flow cytometry analysis. The representative flow dots analysis prior to and post GSI stimulation was shown. The percentage of (A) Th1 cells (CD4$^+$IFN-γ$^+$), (B) Th2 (CD4$^+$IL-4$^+$), (C) Tregs (CD4$^+$FoxP3$^+$), and (D) Th17 (CD4$^+$IL-17$^+$) was compared between cells with and without GSI stimulation in both sepsis and septic shock. The individual level for each subject was shown. Significance was calculated using paired t-test.

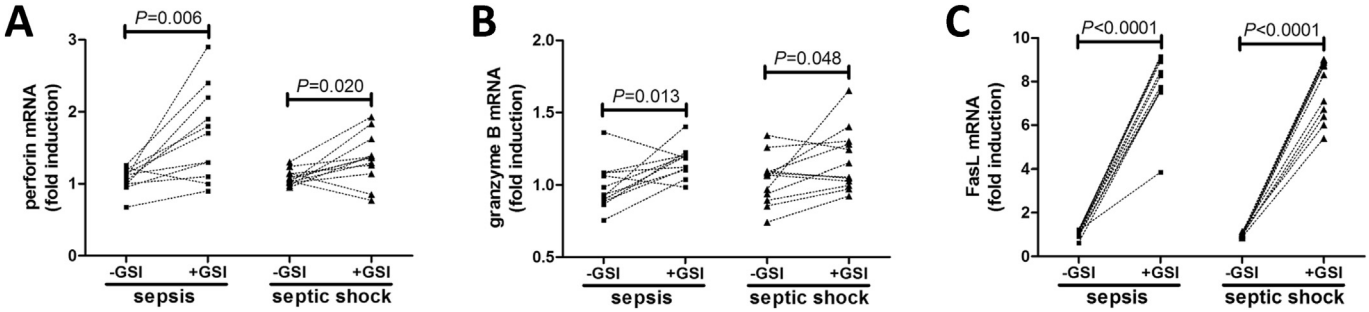

Fig. 6. mRNA relative levels of cytotoxic molecules in CD8$^+$ T cells from sepsis (n = 11) and septic shock (n = 12) in response to Notch signaling inhibition. Purified CD8$^+$ T cells were stimulated with GSI for 24 h. Cells were harvested for real-time PCR analysis. (A) perforin, (B) granzyme B, (C) FasL mRNA relative level in CD8$^+$ T cells was compared between cells with and without GSI stimulation in both sepsis and septic shock. The individual level for each subject was shown. Significance was calculated using paired t-test.

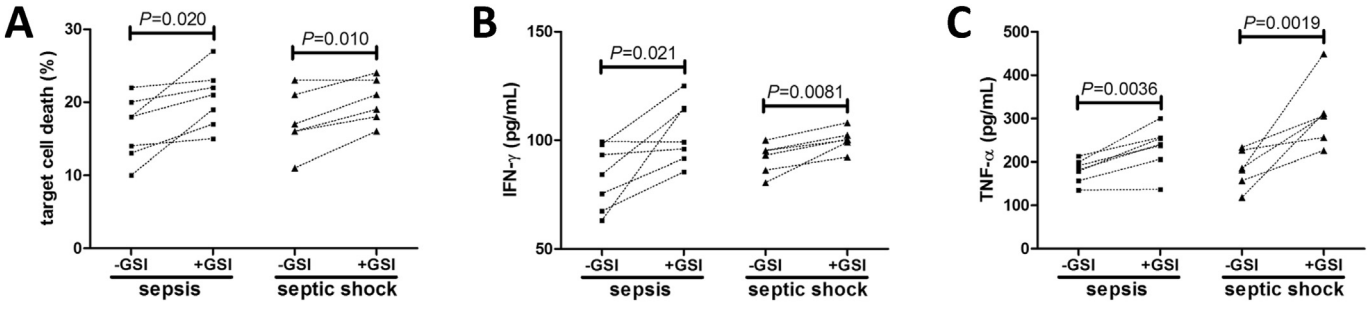

Fig. 7. Target cell death and cytokine production in cocultured system between HLA-A*0201 restricted sepsis (n = 7)/septic shock (n = 6) and HCT-116 cells. Purified CD8$^+$ T cells were stimulated with GSI for 24 h, and were washed twice. 10^5 of stimulated CD8$^+$ T cells were cocultured with 5×10^5 of HCT-116 cells. Supernatants were harvested 48 h post coculture. (A) Target cell death, (B) IFN-γ expression, and (C) TNF-α expression was compared between cells with and without GSI stimulation in both sepsis and septic shock. The individual level for each subject was shown. Significance was calculated using paired t-test.

IL-1β and IL-6 [27]. Notch1 promoted reprogramming of mitochondrial metabolism for proinflammatory macrophage activation [28]. Thus, proinflammatory cytokines secretion and nuclear factor-κB pathway were suppressed in macrophages from myeloid-specific RBP-J$^{-/-}$ mouse [24]. Cecal ligation and puncture operation-induced septic cardiac dysfunction improved myocardial injury by Notch1 deficiency *in vivo*, which presented by the decreased expression of IL-1β and relieved apoptosis of cardiomyocytes [29]. However, the direct modulatory activity of Notch signaling pathway to immune cells, especially to T cell function, was still not fully elucidated in sepsis.

T cell dysfunction and exhaustion was common in cancers and chronic infections [30,31], favoring invasion and metastasis of malignant tumor and viral persistence. Sepsis also induces T cell immunoparalysis [32] and long-term immunosuppression [33] by losing naïve T cells [34], modulating the balance of Th1/Th2 [35] and Treg/Th17 [36], expanding Tregs population/function [33,35], and impairing primary CD8$^+$ T cell responses [37]. We also demonstrated the dysfunctional CD4$^+$ and CD8$^+$ T cells in sepsis, which presented as decreased cell accounts, reduced expression of cytotoxic molecules in CD8$^+$ T cells, impaired cytolytic activity of CD8$^+$ T cells, down-regulation of Th1/Th17 transcriptional factors, but expanded FoxP3 mRNA expression. The decrease of T-bet, RORγt, perforin, granzyme B, and FasL might not a reflection of reduced expression in T cells, but caused by the general reduction in total T cell numbers in the blood of sepsis patients. However, the increased FoxP3 mRNA expression might reveal that decreased T cell accounts might not contribute to the reduction of other transcription factors and cytolytic molecules. Moreover, mRNA expression of CD4$^+$ T cells transcription factor and cytotoxic molecules of CD8$^+$ T cells in CD3$^+$ T cells presented similar trends with those in PBMCs, furthering confirming the dysfunction of T cells in sepsis. Although elevated Notch1 and Notch2 expression modulated effector function and cytokine production by CD4$^+$ and CD8$^+$ T cells [38], the regulatory function of Notch signaling pathway to T cell activity in sepsis has not been previously reported.

It has been revealed that Notch signaling not only enhanced naïve CD4$^+$ T cell sensitivity to antigens [39], but also maintained the survival of memory CD4$^+$ T cells and enhanced activation, proliferation, and anti-apoptotic signals of differentiated CD4$^+$ T cells [40–42]. Several studies provided evidence that NICD induced Th1 and Th2 differentiation by binding and activating of T-bet and GATA-3 *in vivo* [41]. Moreover, Notch signaling also induced pathologic Th17 and Th22 responses to viral infections [18,43].

In contrast, our present study showed that Notch signaling inhibition increased IFN-γ and IL-17 secretion by CD4$^+$ T cells in sepsis and septic shock patients, indicating that Notch signaling pathway promoted Th1 and Th17 effector function within CD4$^+$ T cells in sepsis. However, Notch signaling might not influence Th2 and Tregs function in sepsis. This was partly consistent of the previous report by Pan et al. that inhibition of Notch signaling pathway dampened PD-1 expression on CD4$^+$ T cells and IL-10 expression by CD4$^+$ T cells in sepsis-induced immunosuppression [44]. Thus, the role of Notch acts as promotor or inhibitor of CD4$^+$ T cell function might depend on signals of the environment, such as cytokines and disease status. Elevated Notch1 signaling might induce CD4$^+$ T cell dysfunction in sepsis and septic shock.

Notch signaling regulated $CD8^+$ T cells through multiple ways, such as direct up-regulation of perferin and granzyme B, enhancement of differentiation toward short-lived effector cells, and maintenance of memory T cells [45]. However, Notch signaling pathway dampened both peripheral and tissue-resident $CD8^+$ T cell activity in immunosuppressive status, such as colorectal carcinoma [46] and lung adenocarcinma [47]. $CD8^+$ T cells exerted cytolytic activity *via* two independently functioning but complementary pathways: perforin-granzyme molecules and Fas/FasL interaction [47]. Notch signaling inhibition elevated mRNA levels of perforin, granzyme B, and FasL in $CD8^+$ T cells from sepsis and septic shock. Furthermore, the suppressive cytolytic function of $CD8^+$ T cells from sepsis and septic shock patients was recovered by Notch signaling inhibition. Thus, elevated Notch1 signaling might also contribute to $CD8^+$ T cell dysfunction in sepsis and septic shock.

In conclusion, peripheral high level of Notch1 might suppress differentiation of $CD4^+$ T cells and cytolytic activity of $CD8^+$ T cells in sepsis. This critical function of Notch signaling pathway to T cell dysfunction might provide novel therapeutic strategies for treatment of sepsis.

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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