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ORIGINAL ARTICLE

Effect of interleukin-17 on *in vitro* cytokine production in healthy controls and patients with severe sepsis



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KEYWORDS

cytokine response;
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Background/Purpose: Interleukin (IL)-17 family members (IL-17A to IL-17F) are appearing to play key roles in host defense and inflammatory disease. Recently, several cytokines, such as IL-6, IL-10, IL-12, and transforming growth factor (TGF)- β 1, were shown to have vital roles in severe sepsis. However, the influence of IL-17 on these cytokine responses from peripheral blood mononuclear cells (PBMCs) is unclear.

Methods: Fifty-two patients who were admitted to our intensive care unit (ICU) because of severe sepsis were enrolled into this study. To validate experimental findings, 25 healthy controls were enrolled. Lipopolysaccharide-stimulated PBMCs with IL-17 or anti-IL-17 treatments were cultured for 24 hours. IL-6, IL-10, IL-12, and TGF- β 1 levels in supernatants were measured.

Results: The IL-12 production from stimulated PBMCs was increased after IL-17 treatment in both control and patient groups. Additional treatment of anti-IL-17 enhanced IL-10 production but decreased IL-12 production from stimulated PBMCs of healthy controls and patients with severe sepsis.

Conclusion: IL-17 was helpful for inflammation in severe sepsis. Lack of IL-17 decreased IL-12 and enhanced IL-10 production from PBMCs, which resulted in immune imbalance.

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Conflicts of interest: We confirm that no author has any financial or personal relationships with other organizations that could inappropriately influence this work.

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Introduction

Severe sepsis is the first cause of death in medical intensive care units (ICUs). It is now thought that a hyperinflammatory response at the beginning is designed to eradicate the pathogens and immune dysfunction results in death.^{1–3} The peptidoglycan of gram-positive bacteria can bind to toll-like receptor (TLR)-2 and the lipopolysaccharide (LPS) of gram-negative bacteria binds to TLR-4. In immune cells, cytosolic nuclear factor kappa-B (NF- κ B) is activated through TLR-2 and TLR-4, and this enhances cytokine production. CD4⁺ T help cells play an important role in severe sepsis. Naïve CD4⁺ T helper (Th) cells now can differentiate toward Th1, Th2, Th17, and regulatory T (T_{reg}) phenotypes according to different cytokine induction.⁴ A combination of interleukin (IL)-6 and transforming growth factor (TGF)- β skews toward Th17.⁵ Th17 cells can produce IL-17A and IL-17F, which behaves as a potent inflammatory cytokine.⁶ Many cells express IL-17A and IL-17F including Th17 cells, natural killer (NK) cells, mast cells, and neutrophils. Th17 cells are the main cells to produce IL-17A and IL-17F.⁷ IL-17 family members (IL-17A to IL-17F) also play key roles in host defense and inflammatory disease.

IL-17A and IL-17F can promote innate immunity by triggering proinflammatory cytokine response. IL-17 stimulated human macrophages to produce and express IL- β and tumor necrosis factor (TNF)- α .⁸ Recently, Flierl et al⁹ reported an important animal study. They found that neutralization of IL-17 improved sepsis. It still worked even though the anti-IL-17A administration was delayed for 12 hours. Furthermore, IL-17 antagonist significantly suppressed plasma TNF- α , IL-1 β , and IL-6 levels, and increased plasma IL-10 and TGF- β levels after cecal ligation and puncture (CLP). IL-17 stimulated lung endothelial cells to produce IL-8, which enhance neutrophil but not lymphocyte chemotaxis.¹⁰ Moreover, IL-17 activated endothelial cells by way of the endothelial adhesion marker expression in a p38 mitogen-activated protein kinase-dependent pathway.

Recently, several cytokines, such as IL-6, IL-10, IL-12, and TGF- β 1, showed vital roles in severe sepsis.^{11–13} Plasma IL-6 and TGF- β 1 levels were associated with disease severity. Plasma IL-10 levels and restored IL-12 responses from peripheral blood mononuclear cells (PBMCs) were associated with mortality. However, the effect of IL-17 on these cytokine responses from PBMCs remains unclear. Thus, an *in vitro* human study was designed to address this and to answer this question. LPS was used as a stimulator because most pathogens in severe sepsis are gram-negative bacteria.

Materials and methods

Study participants

From August 2009 to July 2011, 52 patients with severe sepsis were enrolled in this study after admission to our ICU. Patients with high-dose corticosteroid use, neutropenia, and human immunodeficiency virus infection were excluded. Severe sepsis was defined according to the consensus criteria.^{3,14} Disease severity was evaluated by the Acute Physiology and Chronic Health Evaluation

(APACHE) II score.¹⁵ All patients were treated according to guidelines.^{16,17} Informed consent was provided by a close family member. This investigation (97-2179B) was approved by the Institutional Review Board at Chang Gung Memorial Hospital, Keelung, Taiwan. All comorbidities and histories of the study participants were recorded. Survivors were defined as alive for 28 days. For validating experimental findings, 19 men and 6 women were enrolled as healthy controls from the individuals at health evaluation center.

Plasma and PBMCs preparation

Briefly, 10 mL of whole blood was sampled within 48 hours from each patient and mixed with heparin immediately. Plasma from 2 mL of whole blood was obtained, and stored at -80°C . The PBMCs were separated from residual 8 mL of whole blood by the differential centrifugation using Ficoll-Plaque (Amersham Biosciences, Uppsala, Sweden) within 2 hours.

Flow cytometric analysis of PBMCs

Flow cytometry to detect monocytes, CD4⁺ lymphocytes, CD8⁺ lymphocytes, Th1 cells, Th2 cells, Th17 cells, and T_{reg} cells was performed according to our previous protocols.^{12,18} Data were analyzed with Kaluza software version 1.1 (Beckman Coulter, Brea, CA, USA).

Cell culture

The 5×10^5 PBMCs were plated in four wells of a flat-bottomed 24-well plate (Nunc, Aarhus, Denmark) in 1 mL sterile Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium containing 5% heat-inactivated bovine serum, 1 mM of L-glutamine (Gibco, Grand Island, NY, USA) and 1 mM sodium pyruvate. The cells in the first well (C group) were not stimulated. The cells in the second well (LPS group) were stimulated with 1 $\mu\text{g}/\mu\text{L}$ LPS (Sigma-Aldrich, St. Louis, MO, USA). The cells in the third well (LPS + anti-IL-17 group) were stimulated with 1 $\mu\text{g}/\mu\text{L}$ LPS and treated with 12,500 $\mu\text{g}/\text{mL}$ of neutralizing monoclonal anti-human IL-17A antibody (eBioscience, San Diego, CA, USA). The dose of neutralizing monoclonal anti-human IL-17A is based on our previous work that showed the range of IL-17 response was 0–47.3 $\mu\text{g}/\text{mL}$.¹² The cells in the fourth well (LPS + rhIL-17 group) were stimulated with 1 $\mu\text{g}/\mu\text{L}$ LPS and treated with 100 $\mu\text{g}/\text{mL}$ of recombinant human (rh) IL-17A/F (eBioscience). The plate was incubated at 37°C in 5% CO₂ for 24 hours. Supernatants of the culture wells were retrieved and stored at -80°C .

Cytokine measurement

IL-10 levels in supernatants were assessed with a human enzyme-linked immunosorbent assay (ELISA) kit (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's instructions. IL-6 and TGF- β 1 levels in supernatants were assessed with human ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA). IL-12 levels of supernatants were assessed with a human ELISA kit (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Data were analyzed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Differences for continuous variables between survivors and nonsurvivors were analyzed by Mann–Whitney *U* test. Differences of categorical variables were compared using the Chi-square test. Differences between treatment and no treatment in the same subjects were analyzed by Wilcoxon signed-rank test. A *p* value < 0.05 was considered statistically significant.

Results

The clinical characteristics of healthy controls and patients with severe sepsis are shown in Table 1. The mean age of patients was higher than that of controls. Plasma IL-6, IL-10, and IL-17 levels in patients with severe sepsis were significantly higher than those in healthy controls. Plasma

IL-12 and TGF- β 1 levels were similar between control and patient groups. There was no difference in age, sex, APACHE II score, history, and adverse event between survivors and nonsurvivors with severe sepsis. Plasma IL-6 level in nonsurvivors was higher than that in survivors. Plasma IL-10, IL-12, IL-17, and TGF- β 1 levels were similar among survivors and nonsurvivors.

Table 2 shows cell analysis in PBMCs between healthy controls and patients with severe sepsis. Compared with controls, percentages of CD8⁺ and CD4⁺ lymphocytes in PBMCs of patients were significantly decreased. Monocyte percentage in PBMCs was similar among controls and patients. Percentage of Th17 cells in CD4⁺ lymphocytes in patients was higher than that in controls. Percentages of Th1, Th2, and T_{reg} cells in CD4⁺ lymphocytes did not differ between the control and patient groups.

IL-6 production from PBMCs was significantly increased after LPS stimulation (Fig. 1) in both control and patient

Table 1 Clinical characteristics of healthy controls and patients with severe sepsis (number, mean \pm standard error of the mean).

	Controls (<i>n</i> = 25)	All patients (<i>n</i> = 52)	Survivors (<i>n</i> = 38)	Nonsurvivors (<i>n</i> = 14)
Age (y)	60.72 \pm 2.04	72.31 \pm 1.72*	71.11 \pm 2.10	75.57 \pm 2.79
Male (%)	19 (76)	28 (54)	22	6
APACHE II score		24.38 \pm 0.86	23.39 \pm 0.96	27.07 \pm 1.71
History (%)				
COPD		8 (15)	4 (11)	4 (29)
Heart failure		2 (4)	2 (5)	0 (0)
Pneumoconiosis		3 (6)	1 (3)	2 (14)
Hypertension		24 (46)	17 (45)	7 (50)
Diabetes mellitus		24 (46)	18 (47)	6 (43)
Old CVA		22 (42)	17 (45)	5 (36)
ESRD		8 (15)	5 (13)	3 (21)
Liver cirrhosis		2 (4)	1 (3)	1 (7)
Active malignancy		3 (6)	2 (5)	1 (7)
Infection source				
Pneumonia		44 (85)	33 (87)	11 (79)
UTI		3 (6)	3 (8)	0 (0)
Other		5 (9)	2 (5)	3 (21)
Adverse event				
New arrhythmia		4 (8)	3 (8)	1 (7)
GI bleeding		11 (21)	9 (24)	2 (14)
Acute renal failure		22 (42)	14 (37)	8 (57)
Shock		28 (54)	19 (50)	9 (64)
Thrombocytopenia		16 (31)	11 (29)	5 (36)
Jaundice		5 (10)	2 (5)	3 (21)
Bacteremia		15 (29)	11 (29)	4 (29)
Plasma level, pg/mL				
IL-6	4.23 \pm 2.56	179.87 \pm 24.89 ^a	145.60 \pm 26.71	272.91 \pm 51.06 ^b
IL-10	6.50 \pm 2.03	98.14 \pm 29.20 ^a	92.82 \pm 30.59	112.58 \pm 71.94
IL-12	81.73 \pm 20.50	106.80 \pm 24.76	121.32 \pm 33.41	67.37 \pm 11.94
IL-17	0.97 \pm 0.67	3.59 \pm 1.11 ^a	3.12 \pm 1.06	4.85 \pm 3.01
TGF- β 1	3384.06 \pm 358.04	3015.33 \pm 209.97	3200.20 \pm 263.02	2513.52 \pm 285.43

APACHE = Acute Physiology and Chronic Health Evaluation; COPD = chronic obstructive pulmonary disease; CVA = cerebral vascular accident; ESRD = end-stage renal disease; GI = gastrointestinal; IL = interleukin; TGF = transforming growth factor; UTI = urinary tract infection.

^a *p* < 0.05 compared with controls by Mann–Whitney *U* test.

^b *p* < 0.05 compared with survivors by Mann–Whitney *U* test.

Table 2 Cell analysis in peripheral blood mononuclear cells between healthy controls and patients with severe sepsis (mean ± standard error of the mean).

	Controls (n = 25)	Patients (n = 52)
Monocytes, %	4.03 ± 0.36	5.53 ± 0.76
CD8 ⁺ lymphocytes, %	9.32 ± 1.15	2.67 ± 0.35 ^a
CD4 ⁺ lymphocytes, %	18.54 ± 1.33	5.59 ± 0.75 ^a
Th1 cells in CD4 ⁺ cells, %	59.49 ± 3.61	50.20 ± 3.38
Th2 cells in CD4 ⁺ cells, %	8.83 ± 1.39	9.96 ± 1.29
Th17 cells in CD4 ⁺ cells, %	8.08 ± 2.26	21.94 ± 2.77 ^a
T _{reg} cells in CD4 ⁺ cells, %	12.00 ± 1.00	11.22 ± 1.26

Th = T helper; T_{reg} = regulatory T.

^a *p* < 0.05 compared with controls by Mann–Whitney *U* test.

groups. Additional anti-IL-17 or rhIL-17 treatment did not affect IL-6 production from LPS-stimulated PBMCs in both the control and patient groups.

IL-10 production from PBMCs was significantly increased after LPS stimulation (Fig. 2) in both the control and patient groups. Additional anti-IL-17 treatment significantly increased IL-10 production from LPS-stimulated PBMCs in both the control and patient groups. Additional rhIL-17 treatment did not affect IL-10

production from LPS-stimulated PBMCs in both the control and patient groups.

IL-12 production from PBMCs was significantly increased after LPS stimulation (Fig. 3) in both the control and patient groups. Additional anti-IL-17 treatment significantly decreased IL-12 production from LPS-stimulated PBMCs in both the control and patient groups. Additional rhIL-17 treatment significantly increased IL-12 production from LPS-stimulated PBMCs in both the control and patient groups.

TGF-β1 production from PBMCs was similar among groups of nonstimulation, LPS stimulation, LPS stimulation combined with anti-IL-17 treatment, and LPS stimulation combined with rhIL-17 treatment in both the control and patient groups (Fig. 4).

There was no difference in IL-6, IL-10, IL-12, and TGF-β1 levels from cultured LPS-stimulated PBMCs after anti-IL-17 or rh-IL-17 treatments between survivors and nonsurvivors (Table 3).

Discussion

This study first found that loss of IL-17 resulted in increased IL-10 production from stimulated PBMCs. IL-10 also inhibited Th17 generation in PBMCs culture of healthy donors.¹⁹ Thus, low IL-17 response was associated with IL-10 overproduction, which might further inhibit Th17 generation.

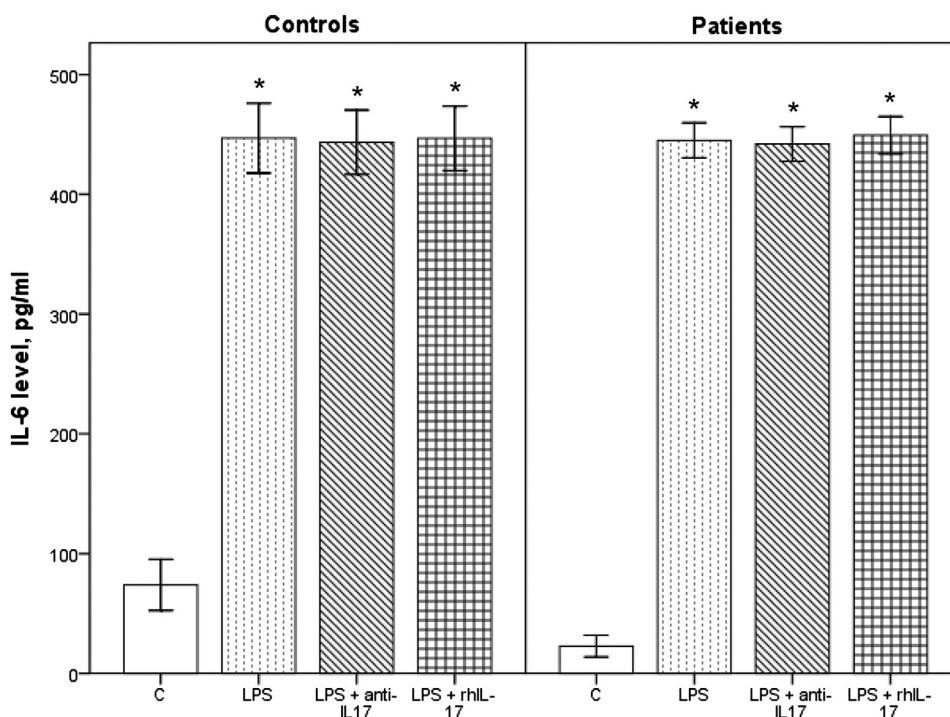


Figure 1 Mean error bars ± one standard error mean of interleukin (IL)-6 level in supernatant of cultured peripheral blood mononuclear cells (PBMCs). Bar C represents nonstimulated cell group. Bar lipopolysaccharide (LPS) represents LPS-stimulated cell group. Bar LPS + anti-IL-17 represents cell group of LPS-stimulation combined with anti-IL-17 treatment. Bar LPS + recombinant human (rh)IL-17 represents cell group of LPS-stimulation combined with rhIL-17 treatment. IL-6 production from PBMCs was significantly increased after LPS stimulation in both control and patient groups. IL-6 production from LPS-stimulated PBMCs was not affected after additional anti-IL-17 or rhIL-17 treatment in both control and patient groups. **p* < 0.05 compared with C group.

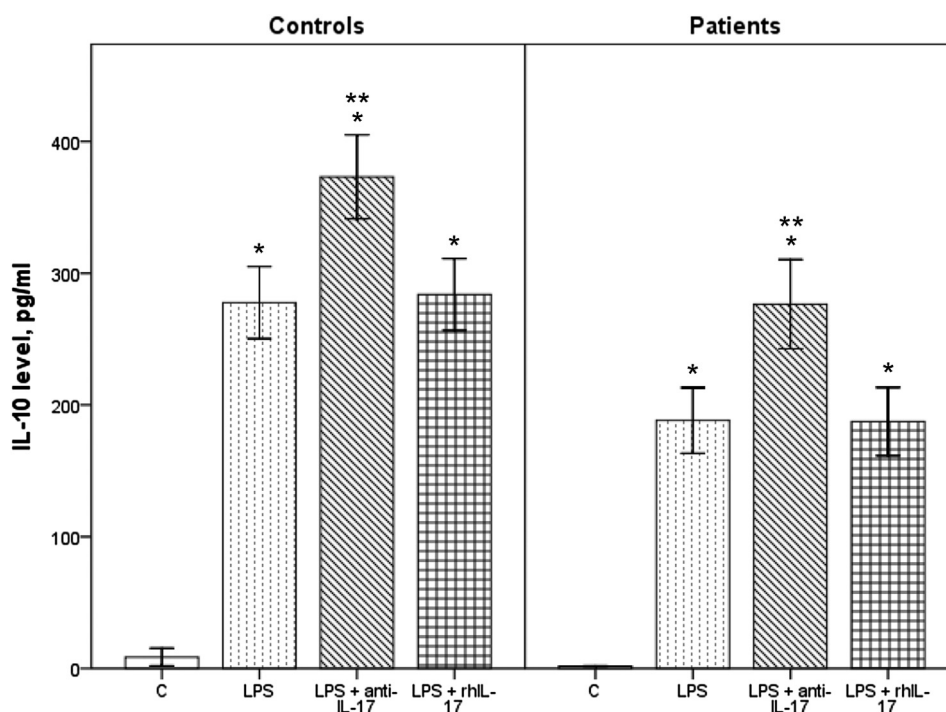


Figure 2 Mean error bars \pm one standard error mean of interleukin (IL)-10 level in supernatant of cultured peripheral blood mononuclear cells (PBMCs). Bar C represents nonstimulated cell group. Bar lipopolysaccharide (LPS) represents LPS-stimulated cell group. Bar LPS + anti-IL-17 represents cell group of LPS-stimulation combined with anti-IL-17 treatment. Bar LPS + recombinant human (rh)IL-17 represents cell group of LPS-stimulation combined with rhIL-17 treatment. IL-10 production from PBMCs was significantly increased after LPS stimulation in both control and patient groups. IL-10 production from LPS-stimulated PBMCs was significantly increased after additional anti-IL-17 treatment in both control and patient groups. IL-10 production from LPS-stimulated PBMCs was not affected after additional rhIL-17 treatment in both control and patient groups. * $p < 0.05$ compared with C group. ** $p < 0.05$ compared with LPS and LPS + rhIL-17 groups.

High IL-10 production from *in vitro* stimulated PBMCs was found in nonsurvivors with severe sepsis.²⁰ Also, persistent high plasma IL-10 level without a decrease was associated with mortality in patients with severe sepsis.¹² All of the aforementioned findings suggest that IL-17 was important in surviving severe sepsis through inhibiting overproduction of IL-10.

In this work, IL-12 production from stimulated PBMCs of patients with sepsis and controls was decreased without the presence of IL-17. Survivors with severe sepsis produced more IL-12 from stimulated PBMCs than nonsurvivors.²⁰ IL-12 production from stimulated PBMCs was also increased after 6 days in survivors with severe sepsis.¹² Most IL-12 is released from activated monocyte/macrophage. IL-12 can enhance native T lymphocyte differentiation to Th1 cells. Th1 cells produce IFN- γ , which modulates activation of monocyte/macrophage and NK cell, stimulates B cells to secrete immunoglobulin, and enhances Th1 cell differentiation. Thus, increase of IL-12 production in patients with severe sepsis may exert a protective effect, which may be related to increased cellular immunity and phagocytic functions. All of the aforementioned factors indicate that patients with severe sepsis might have poor outcome if they lose the help of IL-17.

Although additional IL-17 treatment increased IL-12 production from stimulated PBMCs, the median IL-12 increase with IL-17 treatment was < 10 pg/mL. This

suggests that enhancement of IL-12 production by an increase of IL-17 was limited. IL-17 might also play a negative regulatory role on IL-12 response.²¹ Treatment of IL-17 decreased IL-12-induced IFN- γ production in PBMCs. The decreased IFN- γ production was associated with a 60% decrease in IL-12R β 2 messenger RNA expression. Maybe the cause of low IL-12 increase from stimulated PBMCs after IL-17 treatment was low IFN- γ expression, which results in monocyte/macrophage dysfunction. However, IL-12 significantly decreased IL-17 release from *in vitro* mouse splenocytes in a dose-dependent manner.²² IL-12 and IL-17 might have negatively regulatory roles with regard to each other.

Sepsis deeply disturbs immune balance by inducing an initial systemic inflammatory response and accompanying an anti-inflammatory process as negative feedback. This compensatory anti-inflammatory response secondly becomes harmful as nearly all immune functions are compromised.²³ This prolonged secondary anti-inflammatory response might worsen outcome and played a major role in the decreased defense to nosocomial infections in survivors with sepsis after initial resuscitation and management.

In the study by Flierl et al.,⁹ anti-IL-17A treatment increased survival rate in mice of septic peritonitis by CLP. The protective effect was associated with decreased plasma systemic proinflammatory cytokines and

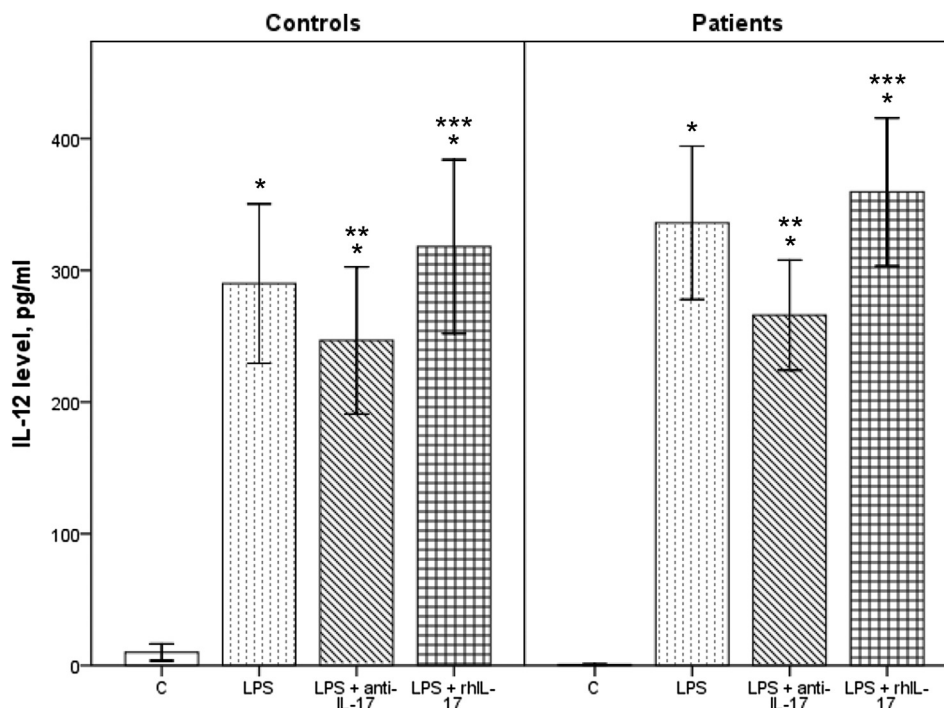


Figure 3 Mean error bars \pm one standard error mean of interleukin (IL)-12 level in supernatant of cultured peripheral blood mononuclear cells (PBMCs). Bar C represents nonstimulated cell group. Bar lipopolysaccharide (LPS) represents LPS-stimulated cell group. Bar LPS + anti-IL-17 represents cell group of LPS-stimulation combined with anti-IL-17 treatment. Bar LPS + recombinant human (rh)IL-17 represents cell group of LPS-stimulation combined with rhIL-17 treatment. IL-12 production from PBMCs was significantly increased after LPS stimulation in both control and patient groups. IL-12 production from LPS-stimulated PBMCs was significantly decreased after additional anti-IL-17 treatment in both control and patient groups. IL-12 production from LPS-stimulated PBMCs was increased after additional rhIL-17 treatment in both control and patient groups. * $p < 0.05$ compared with C group. ** $p < 0.05$ compared with LPS and LPS + rhIL-17 groups. *** $p < 0.05$ compared with LPS and LPS + anti-IL-17 groups.

chemokines. However, CLP significantly reduced the survival rate of IL-17A knockout (KO) mice.²⁴ Compared with wild-type mice, IL-17A KO mice had a more severe degree of acute lung injury. The results of these two studies are conflicting in animal models. Perhaps, in the early stage of sepsis, IL-17A blockade could decrease systemic inflammation and help patient survival after successful initial resuscitation. In the late stage of sepsis, the presence of IL-17 might improve severe sepsis because of decreasing prolonged secondarily anti-inflammatory response by preventing IL-10 overproduction.

In this work, cytokine (IL-6, IL-10, IL-12, and TGF- β 1) levels from cultured LPS-stimulated PBMCs with anti-IL-17 or rh-IL-17 treatments between survivors and nonsurvivors did not differ. This suggests that IL-17 might play a role in the regulating IL-10 and IL-12 from PBMCs. But this regulation after patient admission to the ICU was not related to patient survival. In patients with severe sepsis, absolute circulatory counts of Th17 lymphocytes in survivors were higher than in nonsurvivors.¹⁸ Furthermore, Th17/CD4⁺ ratio and absolute circulatory Th17 lymphocyte counts in survivors were increased after 6 days. Whatever studies are animal or human models, Th17 cells and IL-17 play key roles in sepsis.

There are two limitations that should be highlighted with regard to this study. First, the treatment dose of

rhIL-17 and anti-IL-17 each only had one concentration. The effect of applying different doses of rhIL-17 and anti-IL-17 were not determined. Thus, the dose-dependent effect of IL-17 remains unknown. Second, cytokine responses with rhIL-17 or anti-IL-17 treatments were not measured after 1 week or more of admission. Maybe the effects of rhIL-17 or anti-IL-17 in IL-10 and IL-12 responses from stimulated PBMCs of patients in the late stage of severe sepsis were different from early stage. If the regulation of IL-10 and IL-12 production with IL-17 in the late stage of severe sepsis was associated with final survival, immune therapy regarding IL-17 might become the candidate treatment. Another minor concern is that the mean age of healthy controls is younger than that of patients with severe sepsis. However, the design of this work is a comparison between no treatment and treatment in the same participant. The influence of unequal age between healthy controls and patients with severe sepsis is eliminated.

In conclusion, absence of IL-17 resulted in high IL-10 and low IL-12 production from LPS stimulated PBMCs in patients with sepsis and healthy controls. In patients who survived at the early stage of severe sepsis, loss of IL-17 help might deteriorate the immune imbalance in the late stage. Thus, IL-17 response plays an important role in severe sepsis.

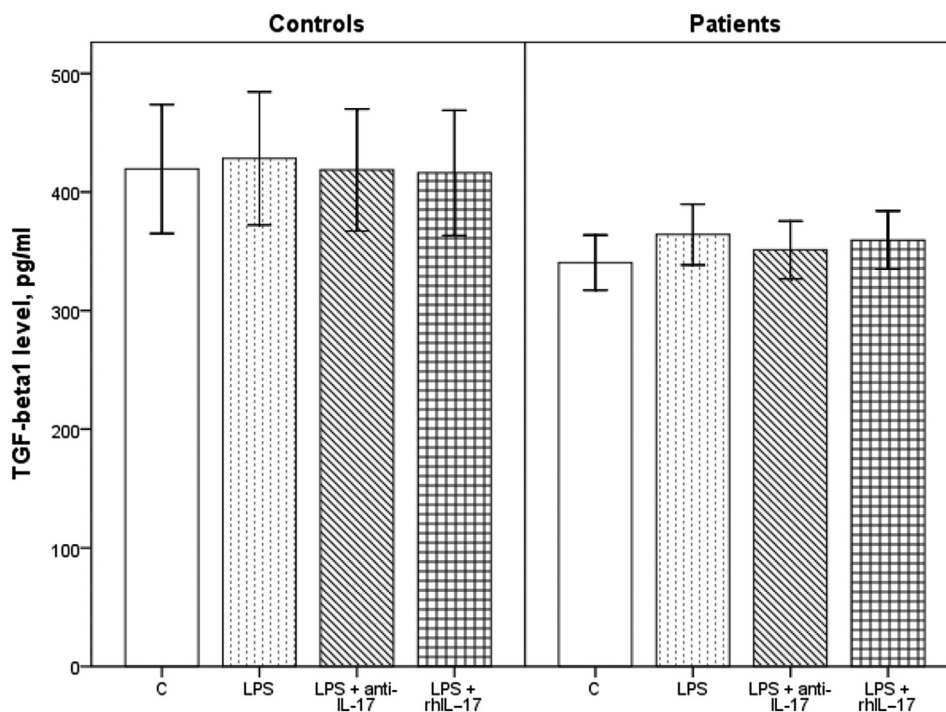


Figure 4 Mean error bars \pm one standard error mean of transforming growth factor (TGF)-beta1 level in supernatant of cultured peripheral blood mononuclear cells (PBMCs). Bar C represents unstimulated cell group. Bar lipopolysaccharide (LPS) represents LPS-stimulated cell group. Bar LPS + anti-IL-17 represents cell group of LPS-stimulation combined with anti-IL-17 treatment. Bar LPS + recombinant human (rh)IL-17 represents cell group of LPS-stimulation combined with rhIL-17 treatment. TGF-beta1 production from PBMCs was not affected by the treatments of LPS, LPS with anti-IL-17, and LPS with rhIL-17.

Table 3 Cytokine levels from cultured lipopolysaccharide-stimulated peripheral blood mononuclear cells after treatments between survivors and nonsurvivors.

Cytokine levels (pg/mL)	Survivors (n = 38)	Nonsurvivors (n = 14)
IL-6		
Anti-IL-17	426.97 \pm 15.36	482.93 \pm 32.44
rhIL-17	435.53 \pm 16.67	486.97 \pm 34.35
IL-10		
Anti-IL-17	272.71 \pm 34.93	286.58 \pm 84.99
rhIL-17	182.67 \pm 26.20	199.90 \pm 66.77
IL-12		
Anti-IL-17	251.79 \pm 50.49	304.34 \pm 74.71
rhIL-17	341.01 \pm 65.94	409.49 \pm 110.43
Transforming growth factor- β 1		
Anti-IL-17	357.98 \pm 30.43	332.85 \pm 30.00
rhIL-17	356.20 \pm 29.61	368.40 \pm 44.35

IL = interleukin; rh = recombinant human.

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