

CD28 monoclonal antibody improve sepsis mortality by amending Th17/Treg balance

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March 07, 2024

Abstract

Background: Sepsis is still developing exorbitantly high mortality. T cell activation plays a crucial role in sepsis's initial innate immune reactions. The imbalance between Th17 and Treg participates in sepsis progression. CD28 signaling pathway was essential for the expression of inflammatory cytokines related to Th17, and play a crucial role in the maintenance of Treg. Methods: 60 sepsis patients' baseline conditions were recorded, and the expressions of inflammatory factors in the peripheral blood and levels of procalcitonin (PCT) were detected. PBMCs were separated, subtypes of T cells and related biomarkers were measured by FACS. Furthermore, the relationship between the above indicators and patients' condition scoring (APACHEII and SOFA) and ICU hospitalization time were analyzed. To investigate effects of CD28 on the balance of Th17 between Treg, anti-CD28 antibody was intraperitoneal administrated to cecal ligation and puncture (CLP) mice. Results: Compared with septic patients who stay in ICU more than 14 days, the Th17/Treg ratio of patients fewer than 14 days was significantly lower. Moreover, the expression of CD28 was significantly higher in sepsis patients than that of healthy donors. After administration of CD28 monoclonal antibody, 7-day mortality and clinical score were significantly improved in septic mice, with splenocyte Th17/Treg ratio decreased. CD28 antibody alleviates the expression of pro-inflammatory factors and spleen injury related to apoptosis. Conclusions: Th17/Treg ratio revealed septic patient severity and as a predictor of ICU stay. CD28 monoclonal antibody could improve 7-day mortality of septic mice by decreasing T cell apoptosis and amending the ratio of Th17/Treg.

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Abstract

Background: Sepsis is still developing exorbitantly high mortality. In response to microbial molecules, T cell activation plays a crucial role in sepsis's initial innate immune reactions. The imbalance between Th17 and Treg participates in sepsis progression. CD28 signaling pathway was essential for the expression of inflammatory cytokines related to Th17, and play a crucial role in the maintenance of Treg. We investigated the correlations of the balance between Th17 and Treg to prognosis in sepsis patients and influence of anti-CD28 antibody on the ratio of Th17 to Treg in sepsis mice.

Methods: 60 sepsis patients' baseline conditions were recorded, and the expressions of inflammatory factors in the peripheral blood and levels of procalcitonin (PCT) were detected. PBMCs were separated, subtypes of T cells and related biomarkers were measured by Fluorescence-activated cell sorting (FACS). Furthermore, the relationship between the above indicators and patients' condition scoring (APACHEII and SOFA) and ICU hospitalization time were analyzed. To investigate effects of CD28 on the balance of Th17 between Treg, anti-CD28 antibody was intraperitoneal administrated to cecal ligation and puncture (CLP) mice.

Results: Compared with septic patients who stay in ICU more than 14 days, the Th17/Treg ratio of patients with ICU hospitalization of fewer than 14 days was significantly lower. The sepsis patients with higher expression of CD28 in peripheral blood lymphocytes have lower APACHE II and SOFA scores. Moreover, the expression of CD28 was significantly higher in sepsis patients than that of healthy donors. After administration of CD28 monoclonal antibody, 7-day mortality and clinical score were significantly improved in septic mice, with splenocyte Th17/Treg ratio decreased. CD28 antibody alleviates the expression of pro-inflammatory factors and spleen injury related to apoptosis.

Conclusions: Th17/Treg ratio revealed septic patient severity and can be used as a predictor of ICU stay. CD28 monoclonal antibody could improve 7-day mortality of septic mice by decreasing T cell apoptosis and amending the ratio of Th17/Treg.

Keywords: Sepsis, length of stay in ICU, Th17/Treg, CD28, Risk stratification, Clinical outcomes

Trial registration: CHEC2019-133, CTR20191855. Registered 27 August 2019

Background

Sepsis is a significant public health concern; the estimated number of sepsis cases each year worldwide has doubled compared with previous estimations and is almost 49 million now[1]. Sepsis refers to the life-threatening organ dysfunction caused by the dysregulation of the body's response to infection, especially immune dysregulation with pro-inflammatory and anti-inflammatory imbalance[2]. The complicated collapsed immune response to severe infection was considered the central pathogenesis of sepsis[3-5].

Meanwhile, immunosuppression also coincides with lymphopenia and loss of immune function. T lymphocytes are one of the critical immune cells with regulating antimicrobial phagocytic and cytotoxic activity. There are many different subtypes T lymphocytes such as naïve T cells, helper T (Th) cells, memory T cells, regular T (Treg) cells (CD4+CD25+ regulatory T cells) and so on with different surface markers, transcriptional regulators, effector molecules and functions. The subtypes of each cell and the ratio between subtypes are different at different stages of T cell immunity[6]. Among such subtypes, Treg and CD4+IL-17+ T helper cells(Th17) share a common precursor cell (the naïve CD4 T cell) and change appearance with the disease progressing[7, 8]. Mice that knocked out the FoxP3 gene, which Treg cell-specific marker controls the T cells development and their regulatory activities, developed fatal inflammatory diseases[9]. Th17, secreting IL-17 and specific transcriptional regulator ROR- γ t, plays an essential physiological role at mucosal barriers and is involved in inflammatory responses to pathogens [10]. The balance between Th17 and Treg has emerged as a prominent factor in regulating autoimmunity[11]. Accumulated evidences suggest that the imbalance of Th17 and Treg is associated with the development of many diseases[12], such as primary Sjögren's syndrome[13], Experimental Autoimmune Encephalomyelitis[14], human graft-versus-host disease[15] and asthma[16].

The ratio of Th17/Treg might become predictors of immune status; the identification and intervention of T cell immune response process in sepsis may be the focus of future treatment. CD28 signal pathway, one of the critical costimulatory molecules on T cells, can active and maintain T cell functions, stimulate

the body's immune response through combining with CTLA-4 and B7(CD80/CD86) molecule on antigen-presenting cells (APC) [17]. CD28 signaling is essential for effector T cells to overcome Treg cell-mediated immunosuppression[17]. CD28 signaling pathway plays a crucial role in maintaining Treg pool size and each Treg subset's homeostasis by promoting the development and proliferation of these cells [18, 19]. Then we observed the ratio of Th17/Treg in predicting severity and outcomes of septic patients, furthermore as the possible hint of the immunotherapy timing. Effects of CD28 signaling pathway on the balance of Th17/Treg in septic mice need to be identified, then providing a therapeutic reference for sepsis.

Methods

Patient characteristic

Patients with sepsis were recruited and the study was approved by the human ethics committee of Changhai Hospital (CHEC2019-133), as preliminary trial of sepsis treatment(CTR20191855), informed consent from all of the subjects or their relatives was obtained before enrollment. 65 patients suffering from sepsis were admitted to the intensive care unit (ICU) in Shanghai Changhai Hospital from January to October 2020, meanwhile 13 healthy volunteers, gender, age and ethnicity was the same, as the control group were recruited. The sepsis diagnostic criteria according to the definitions for sepsis and septic shock established by The Third International Consensus (Sepsis-3)[20]. Eligibility criteria for enrollment in this study included the sepsis patients of either sex by age 18-80 years with systolic blood pressure <80 mmHg, presenting within 8 h of sepsis after taking proper consent from the patient or relatives. The severity of the patients was assessed by Acute Physiology and Chronic Health Evaluation score (APACHE II) and the extent of development of organ dysfunction was assessed by the Sequential Organ Failure Assessment score (SOFA; range, 0-24). Exclusion criteria included: 1. age <18 years; 2. severe acute head injury (GCS score <5); 3.irresuscitable terminal stage at presentation; 4. autoimmune disease (such as rheumatoid arthritis, systemic lupus erythematosus, asthma, or multiple sclerosis); 5. AIDS; 6. acute stroke, myocardial infarction or recent viral hepatitis;7.use of hormones or immunosuppressors within the past 3 months before hospitalization;8.transplant surgery;9.unexpected termination of CBP treatment;10.Patients who resuscitated with anti-inflammatory drugs or corticosteroids; 11. septic patients, of whom blood sampling was not taken within the first 8 h after definition sepsis.

Blood samples and preparation

Venous blood samples were drawn into ethylenediaminetetraacetic acid tubes within 24 hours after the patient is diagnosed with sepsis, isovolumetric venous blood samples were collected from healthy volunteers in the physical examination center of our hospital. Blood samples were refrigerated at 4degC after EDTA anticoagulation. Density gradient centrifugation was conducted at 2,000 rpm for 20 minutes to isolate Peripheral blood mononuclear cells(PBMCs), which were used to detect the expression of membrane markers, and plasma, which was stored at -80degC for subsequent cytokine detection. The concentration of PBMCs was adjusted to 1×10^6 /mL in RPMI 1640 culture solution supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM/L-glutamine, and 10% fetal calf serum (Gibco). The cell suspension was seeded onto 12-well cell culture plates. Cells were treated with 1ul Leukocyte Activation Cocktail, with BD Golgi-Plug(BD), and incubated in darkness at 37°C under 5%-CO₂ atmosphere for 6 hours.

Animals

Male C57BL/6 mice aged 6-8 weeks and weighing 20-25g were used and bred carefully in the Changhai Hospital Animal Laboratory Centre under the ethical guidelines of the National Institutes of Health on Animal Care. Mice were raised in specific pathogen-free cages and maintained at temperatures between 20 and 25 °C and relative humidity of 50-70%. The experimental protocols were approved by the Animal Care and Use Committee of Navy Medical University, Shanghai.

Reagents

Mouse TNF-a, IL-17A, IL-10, IL-6 enzyme-linked immunosorbent assay (ELISA) kits, CD28 functional antibody, fluorescent-labeled monoclonal antibodies anti-mouse CD4-FITC, anti-mouse IgG1-FITC, anti-

mouse CD25-PE, anti-mouse IgG1-PE, anti-mouse FoxP3-APC, anti-mouse IFN-gamma-PE, anti-mouse IgG1-APC, and anti-mouse IL-17-APC were bought from eBiosciences (San Jose, CA, USA). FIX & PERM medium from Invitrogen, USA. Leukocyte Activation Cocktail from BD, Catalog No.550583, contain PMA(Phorbol 12-Myristate 13-Acetate), a calcium ionophore (Ionomycin) and the protein transport inhibitor BD GolgiPlug (Brefeldin A).

Animal Treatment and cecal ligation and puncture (CLP)

Mice were anesthetized with isoflurane and a midline abdominal incision was made. The cecum was mobilized, ligated below the ileocecal valve, and punctured twice with a 22 gauge needle to induce polymicrobial peritonitis. Sham-operated mice underwent the same procedure, including opening the peritoneum and exposing the bowel, but without ligation and needle perforation of the cecum. After surgery, the mice were injected with 1 mL physiologic saline solution for fluid resuscitation and 20ug butorphanol for post-operative analgesia. All mice had unlimited access to food and water both pre- and post-operatively, allowed to die spontaneously from sepsis. According to the literature and our previous research, 5 mg/kg CD28 functional antibody was administered intraperitoneally at the same time[21]. Mice were scored with murine sepsis severity scale and survival rate by two independent, blinded researchers.

Mouse clinical score after treatment.

After CLP-induced polymicrobial sepsis successfully in mice, all of them moved free and none needed analgesia for pain immediately. The clinical scores of mice at 0h, 2h, 6h, 12h, 24h, 48h, 72h, 4d, 5d and 7d by incorporating to the Murine Sepsis Score (MSS) were evaluated. The scoring criteria are as follows: spontaneous activity, response to touch and auditory stimuli, posture, respiration rate and quality (labored breathing or gasping), and appearance (i.e., degree of piloerection), each of these scores variables between 0-4[22].

Spleen index

After the mice were completely anesthetized by sevoflurane on the third day after CLP, mice were weighed and mice's spleen was harvested then measure the length. Spleen index can be expressed as the weight of the spleen divided by the weight of the mouse.

Histology

At 3 days after CLP challenge[21], mice were sacrificed by CO₂ inhalation. Then, the spleens were collected and fixed in 4% paraformaldehyde solution for more than 48 hours before being embedded in paraffin and sectioned. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin (H&E). The congestion and necrosis of the spleen tissue and the aggregation of inflammatory cells were evaluated under an optical microscope. Then the spleen injury scores of mice based on the results of H&E[23] was evaluated by two independent, blinded researchers.

Culture splenocytes

After the mice were completely anesthetized by sevoflurane, the mice were killed by cervical dislocation. Then place the mouse in a bottle filled with alcohol for 5 minutes to sterilize, and operate in a sterile biological operation table: use a sterile instrument to perform laparotomy to remove the spleen tissue of the mouse and place it into a sterile mill previously added with sterile PBS Grind gently in the grinding dish to completely separate the spleen cells, then add erythrocyte lysate and let stand for 10 minutes, then stop with sterile PBS, centrifuge to remove the lower layer of cells. After filtering out the non-cellular components with a filter, count 1×10^6 cells into a sterile culture dish, add Leukocyte Activation Cocktail 1ul and incubate for 6 hours at 37 deg C with 5% CO₂.

Western blot analysis

Proteins from spleen tissues were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were then incubated overnight at 4 degC with antibodies against the protein of interest, including

BCL-2 (D17C4; 3498; Cell signaling), Bax (D2E11; 5023; Cell signaling), GAPDH (D16H11; 5174T; Cell signaling) over-night at 4 degC. Protein quantification was measured in optical density units using Image Lab software (Bio-Rad, CA, USA) and was normalized to the corresponding sample expression of GAPDH.

Analysis of apoptosis

According to the manufacturer's instructions, the induction of apoptosis in the spleen was detected using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)[24]. The apoptotic index was quantified by the proportion of TUNEL-positive cells[25], three parallel experiments were done for each group.

Flow cytometry

All anti-bodies listed were obtained from eBioscience, San Diego, CA, USA, 5ul labeled antibody was added in each procedure. The expression of CD28, CTLA-4, CD80 and CD86 in CD4⁺ T Lymphocytes was assayed in PBMCs and splenocytes. Add anti-human (mouse)CD4-FITC monoclonal antibodies, anti-human(mouse) CD28-PE, anti-human(mouse) CTLA-4-APC or anti-human(mouse) CD80-PE, anti-human(mouse) CD86-APC at room temperature away from light for 30 minutes. A parallel control group was treated with isotype controls according to the manufacturer's recommendations.

Stimulated and cultured mononuclear cells were collected, pre-incubated for 15 minutes with unlabeled isotype control Abs (IgG1 or IgG2b). Then incubated with anti-human CD3-FITC, anti-human CD8-PE, or anti-human CD4-FITC and anti-human CD25-PE, a parallel control group was treated. splenocytes incubated with anti-mouse CD4-FITC, with or without anti-mouse CD25-PE at room temperature away from light for 30 minutes, then incubated at room temperature away from light for 15 minutes after treating with 100 μ L FIX & PERM medium A and B (Invitrogen, USA). Then, anti-human IL-17-APC, anti-human IFN-g-APC, anti-human IL-4-APC or anti-human FoxP3-APC was added in PBMCs and anti-mouse IL-17-APC, anti-mouse IFN-g-APC, anti-mouse IL-4-APC or anti-mouse FoxP3-APC was added in splenocytes. A parallel control group was treated d with isotype controls. Both suspensions were incubated at 4 temperature away from light for one night.

Plasma cytokine

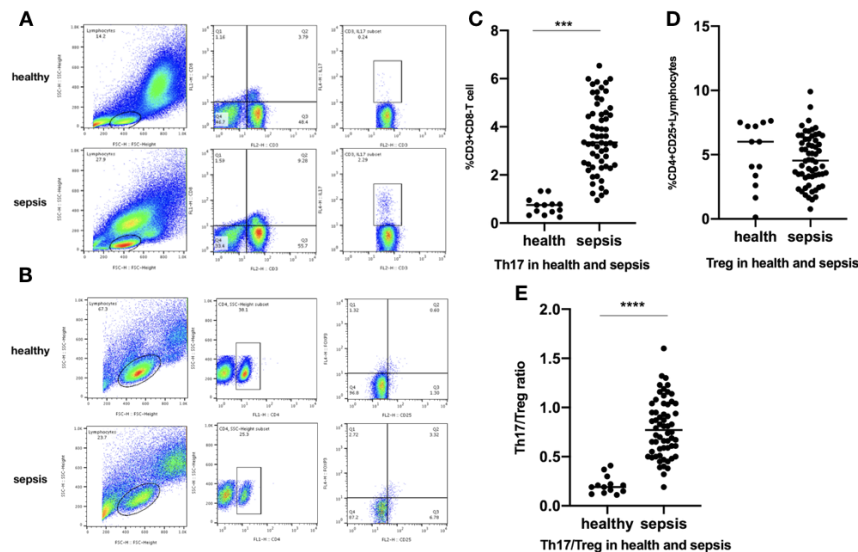
The patients' serum was collected as shown above, and mice blood samples were collected by heart puncture when anesthetized by sevoflurane 3 days after CLP and CD28 challenge. Then, the mice's blood was sent into ethylenediaminetetraacetic acid tube and placed in a centrifuge tube, centrifuge at 12,000 rpm for 5 minutes, take the supernatant, measure the content of TNF-a, IL-17, IL-10, and IL-6 in patients or mice serum according to Elisa manufacturer's recommendations (Thermo Fisher).

Statistics

All analyses were done using SPSS, version 22.0 (IBM Corp. Armonk, NY, USA), MedCalc Statistical Software, version 19.5 (MedCalc Software Ltd, Ostend, Belgium) and GraphPad Prism 8.0(GraphPad Software, San Diego, CA, USA). Continuous variables were reported as mean \pm SD or as median (IQR) after testing their normal distribution using the Kolmogorov-Smirnov test. For two-group comparisons, the independent samples t-test was used for normally distributed data, and the Mann-Whitney test was used for non-normally distributed data. For the comparison of multi-group, one-way analysis of variance and the Kruskal-Wallis test was used to analyze normally and non-normally distributed data. Categorical data are summarized using number (percentage), and were compared using the chi-square or Fisher's exact test. Spearman's rank correlation was applied to determine the correlation between variables. The area under the receiver operating characteristic (ROC) curve was calculated to evaluate the tested parameters' diagnostic and prognostic value and a value of P <0.05 was considered statistically significant.

Results

Sepsis induced Th17 and ratio of Th17/Treg increasing



Th17 and Treg expression on CD4⁺T lymphocytes and their ratio were compared between sepsis patients and volunteers. Although there was no difference in health volunteers and septic patients [4.998(3.474-6.523) vs 4.625(4.117-5.133), P=0.5573], sepsis caused Th17 increased obviously [0.6977 (0.4852-0.9102) vs 3.580(3.209-3.951), P<0.01]. And the ratio of Th17 to Treg higher in sepsis patients [0.2131 (0.1400-0.2600) vs 0.7907 (0.5575-1.038), P <0.01]. This suggest in initial sepsis Th17 cells increase significantly, while Treg which acted as an inflammatory brake remained relatively stable.

Figure 1: **A**, Th17 expression in healthy volunteers and sepsis patients, take CD3 as abscissa and CD8 as ordinate, find out the CD3+CD8-cell group, and then mark IL-17 (ordinate). **B**, Treg expression in healthy volunteers and sepsis patients, take CD4 as the abscissa, find out CD4+cell group, and then mark FoxP3 (ordinate). **C**, Expression of Th17 between health volunteers and septic patients. **D**, Expression of Treg between health volunteers and septic patients. **E**, Expression of Th17/Treg between health volunteers and septic patients, ***, p<0.001, ****, p<0.0001.

Patient characteristics and Comparison

Sixty-five adult patients with sepsis were admitted, including 41 males and 24 females, and 5 cases died in the ICU. According to the length of ICU stay more or fewer than 14 days, 60 patients of survival were divided into two groups. There were no significantly difference in demographic data of two groups (Table 1), except that the median age of patients whose ICU stay more than 14 days was older than that of group of ICU stay fewer than 14 days (63.33±10.41 vs 72.63±7.45, P =0.015), which was in parallel with SOFA score [5.17 (3.00-6.00) vs 8.00 (5.00-11.00), P =0.01] and APACHE II score [11.79 (7.00-14.00) vs 16.62 (13.00-21.00), P =0.01].

Table 1 Baseline levels of sepsis patients with different length of stay in ICU

Parameter	Total	ICU stay<14d (n=36)	ICU stay>14d (n=24)	p
Age, years	67.05±10.35	63.33±10.41	72.63±7.45	0.015
gender(M/F)	39/21	24/12	15/9	0.740
height, cm	166.73±9.46	166.78±9.21	166.67±10.03	0.862
body weight, kg	67.65±13.13	65.64±13.18	70.67±12.73	0.516
White blood cells ,103/μL	18.12(11.09-23.68)	19.00(12.38-24.27)	16.79(8.26-20.95)	0.165

Parameter	Total	ICU stay<14d (n=36)	ICU stay>14d (n=24)	p
Lymphocytes, 103/ μ L	0.79(0.38-0.97)	0.70(0.39-0.91)	0.91(0.34-1.13)	0.868
SOFA score	6.30(4.00-9.00)	5.17(3.00-6.00)	8.00(5.00-11.00)	0.001
APACHE II score	13.72(8.00-17.00)	11.79(7.00-14.00)	16.62(13.00-21.00)	0.001

As showed in Table 2, despite the severer condition of septic patients that ICU stay more than 14 days, neither Th1, Th2, Treg cells subtypes nor their remarkable cytokines were changed obviously when compared with patients that stay fewer than 14 days. However, both the ratio of Th17 and the IL-17 level of septic patients that ICU stay more than 14 days were increased significantly [compared with patients that stay fewer than 14 days, 3.20 (2.21-3.92) vs 4.32 (3.33-5.47), $P=0.002$ and 139.68 (98.53-162.81) vs 169.32 (131.86-206.94), $P=0.02$]. As a result, the ratio of Th17/Treg of septic patients that ICU stay more than 14 days was also increased significantly (compared with patients that stay fewer than 14 days, 0.76 ± 0.31 vs 0.98 ± 0.18 , $P=0.001$, Fig.2a). The ratio of Th17/Treg of septic patients was positively correlated with the length of stay in ICU (Fig.2b) and SOFA score ($r^2=0.2061$, Fig. 2c) and APACHE II score ($r^2=0.2583$, Fig. 2d) when the patients were admitted to ICU.

Table 2 Cytokines in sepsis patients with different length of stay in ICU

Parameter	Total	ICU stay<14d(n=36)	ICU stay>14d(n=24)	p
IL-6(pg/ml)	258.07(44.46-374.59)	202.90(43.65-301.07)	340.83(65.60-376.26)	0.154
TNF-a (pg/ml)	32.47(13.55-34.00)	35.80(13.10-34.28)	27.48(18.05-34.00)	0.415
IL-10(pg/ml)	47.52(8.56-50.80)	52.40(8.38-35.33)	40.19(10.61-57.09)	0.327
IL-8(pg/ml)	1024.47(74.08-1024.47)	1066.58(63.40-1181.35)	961.30(175.50-976.77)	0.284
IL-17(pg/ml)	151.54(107.60-188.90)	139.68(98.53-162.81)	169.32(131.86-206.94)	0.020
PCT	13.37(1.41-16.48)	10.21(1.13-9.99)	18.10(3.115-23.30)	0.097
Th1	14.02(6.91-19.47)	13.43(7.82-15.12)	14.90(5.87-21.70)	0.350
Th2	2.61(1.19-2.96)	1.98(1.08-2.81)	3.55(1.35-3.60)	0.182
Th1/Th2	9.22(3.65-12.78)	9.61(3.48-10.63)	8.63(3.65-14.93)	0.461
Th17	3.65(2.42-4.73)	3.20(2.21-3.92)	4.32(3.33-5.47)	0.002
Treg	4.63(3.31-6.20)	4.62(2.78-6.48)	4.64(3.33-5.85)	0.946
Th17/Treg	0.85 ± 0.28	0.76 ± 0.31	0.98 ± 0.18	0.001

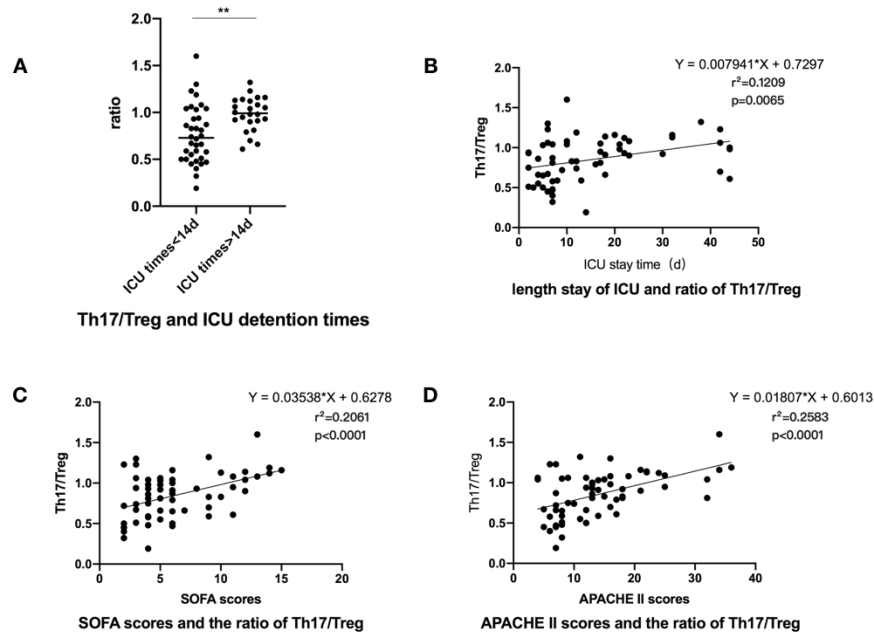


Figure 2. **A:** Comparison of Th17/Treg ratio among patients with different length of hospital stay; **B:** Correlation between Th17/Treg and length of ICU stay; **C:** Correlation between SOFA score and Th17/Treg ratio; **D:** Correlation between APACHE II score and Th17/Treg ratio. SOFA, Sequential Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Assessment II. ** $p < 0.01$.

The predictive of Th17/Treg ratio, SOFA score, APACHE II score on length of ICU stay

The limited data suggested the ratio of Th17/Treg predicted the length of septic patient ICU stay. The area under the ROC curve(AUC)of the Th17/Treg ratio, SOFA score and APACHE II score was 0.747(95%CI: 0.618-0.850), 0.764(95%CI: 0.637-0.864) and 0.760(95%CI: 0.6333-0.861) respectively for ICU stay more than 14 days. Combined either SOFA score or APACHE II score with the ratio of Th17 / Treg, although there is no statistically difference each other, but its AUC of predictive value on the length of sepsis patients' ICU stay is higher, with AUC value of 0.782 (95% CI: 0.656-0.878) or 0.800 (95% CI: 0.676-0.892). The sensitivity and specificity for predicting ICU stay based on cut-off values are shown in Figure 3.

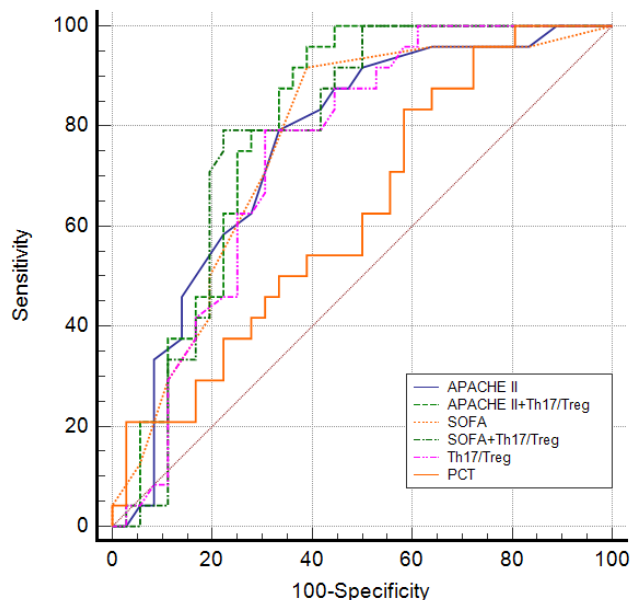


Fig.3- Receiver operating characteristic (ROC) curves for the Th17/Treg ratio, acute physiology and chronic health evaluation (APACHE) II, sequential organ failure assessment (SOFA) and Th17/Treg ratio in combination with APACHE II score, Th17/Treg ratio in combination with SOFA score for predicting sepsis patients more than 14-day ICU stay time. The area under the curve (AUC) demonstrates that the Th17/Treg ratio measures 0.747 (95% CI 0.618 to 0.850), the APACHE II score was 0.760 (95% CI 0.633 to 0.861), the SOFA score measures 0.764 (95% CI 0.637 to 0.864), PCT measures 0.627 (95% CI 0.493 to 0.749), the Th17/Treg ratio in combination with the SOFA score measures 0.782 (95% CI 0.656 to 0.878), and the Th17/Treg ratio in combination with the APACHE II score measures 0.800 (95% CI 0.676 to 0.892).

CD28 and ligands expression levels in sepsis patients

The expression level of CD28 of peripheral blood CD4+T lymphocytes was obviously increased in sepsis patients [compared with healthy volunteers, 84.21 (73.24-95.05) vs 17.05 (12.40-22.10), $p < 0.001$, Figure 4]. Meanwhile neither CD28, CTLA-4 nor their ligands CD80, CD86 expression levels was without significant in all sepsis patients. The Th17/Treg ratio was positively correlated with the expression level of CD28 in CD4+ T lymphocytes ($r^2 = 0.2615$, $P < 0.01$, Figure 4c).

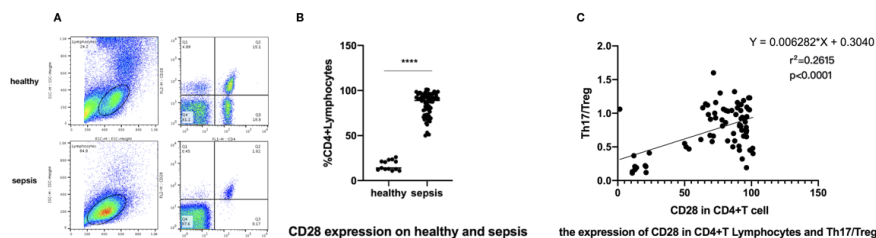


Fig 4. **A**, the expression of Treg in healthy volunteers and sepsis patients. **B**, CD28 molecules expression on CD4+ lymphocyte cells between sepsis and healthy. Compared with healthy. **C**, Th17/Treg ratio correlate with the expression of CD28 in CD4+ lymphocytes. **** $p < 0.001$.

Table 3 Comparison of CD28 expression in sepsis patients with different length of stay in ICU

Parameter	Total	ICU stay<14d(n=36)	ICU stay>14d (n=24)	p
CD28	84.21(73.24-95.05)	85.79(79.50-95.39)	81.84(69.92-92.30)	0.152
CD80	1.39(1.02-1.51)	1.45(1.13-1.61)	1.29(0.80-1.34)	0.145
CD86	52.54(38.48-66.13)	55.93(43.38-67.09)	47.45(34.22-62.89)	0.145
CTLA-4	1.79(0.79-1.70)	1.94(0.81-1.70)	1.56(0.75-1.74)	0.442

Anti-CD28 improved mortality of CLP-induced sepsis mice

Therefore, animal models were designed to verify effects of CD28 on ratio of Th17 to Treg in septic mice. With administrated anti-CD28 functional antibody (5 mg/kg) after CLP, both 7-days survival rate (Fig5.a) and clinical scores (Fig5.b) of mice were improved obviously. The white pulp nodule in septic mice's spleen was disordered, and the boundary between white pulp and red pulp was blurred (Fig5. c). After detur CD28 antibody, the disorder of white pulp nodule and histopathological score (Fig5. d) were alleviated while spleen index (Fig5. e) were aggrandized.

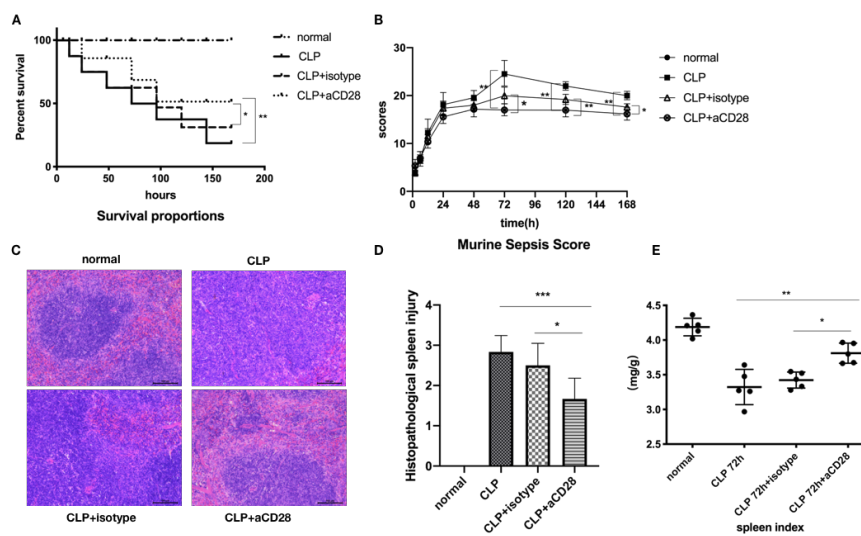


Fig.5 **A** , the Kaplan-Meier survival analysis 7 d after CLP in isotype treat and CD28 functional antibody mice. The Kaplan-Meier survival curves were compared by the log-rank test. Line-graphs show mean and standard error(n=10 mice per group). **B** , Mice's Murine Sepsis Score (MSS) over time that were administered 5 mg/kg CD28 functional antibody (n = 5). **C** , Histology of spleen (Original magnification: 200×), Scale bar, 100 mm. **D** , Spleen histopathological score(n=5), the values presented are mean ±SEM, n=6.**E** , spleen index(n=5). The values presented are mean ±SEM, *p<0.05, ** p<0.01, ***p<0.001, The data are representative of 3 independent experiments.

Anti-CD28 Ab amended Th17/Treg imbalance in sepsis mice

As Th17 and Treg variation in sepsis patients, Th17 and Treg variation in CLP induced sepsis mice had similar trends. Treg and Th17 in spleen of CLP mice were decreased obviously (Figure 6a, b). After treated with CD28 antibody, the extent of Treg and Th17' increase amplitude was lower, as well as the proportion of Th17/Treg (Figure 6c, d, e). Serum levels of IL-6,TNF- α and IL-17A were increased significantly in CLP mice, while the level of IL-10 was increased slightly. Meanwhile, such trends were reversed by administrated with CD28 functional antibody (Fig6. F, G, H and I).

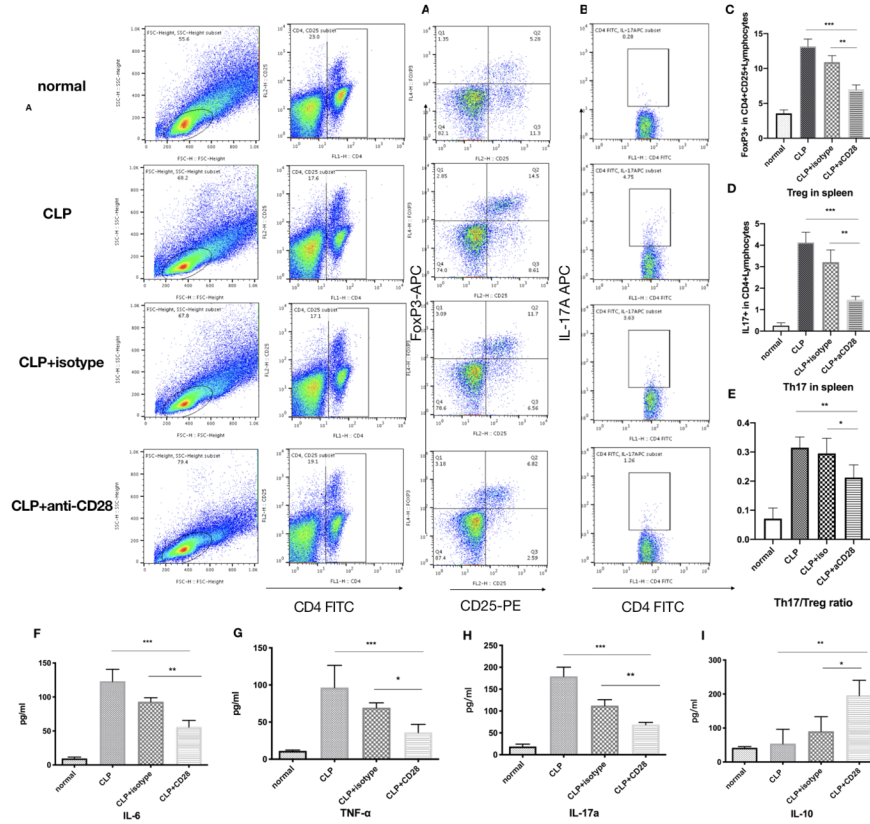


Fig.6 Flow cytometry of Treg cell(A), Th17 cell(B) in spleen after 3 days of CLP. The percentage of Treg(C) and the Th17 (D) in different groups by flow cytometry. E , the ratio of Th17 to Treg is calculated. Expression of serum related inflammatory factors in mice 3 days after CLP, IL-6(F), TNF-a(G), IL-17A(H) and IL-10(I). The values presented are mean \pm SEM (n=5). *p<0.05, **p<0.01, ***p<0.001. The data are representative of 3 independent experiments.

Anti-CD28 reduced CLP-induced splenocytes apoptosis

T lymphocyte subsets differentiation and lymphocytes apoptosis played an important role in septic rats[26, 27]. The proportion of spleen cell apoptosis was significantly increased in CLP mice, with reduced nucleus number, irregular shape of the nucleus, state of condensation or nuclear fragments appear, while anti-CD28 relieved such spleen apoptosis significantly (Fig.7a, b). The BCL-2 and Bax signaling pathways are required in spleen homogenate for apoptosis in CLP-induced septic mice[28]. As illustrated in Figure.7c, expression of the BCL-2 was significantly increased after anti-CD28 challenge (Fig.7d, p<0.01), while expression of the Bax was remarkably decreased (Fig.7e, p<0.01).

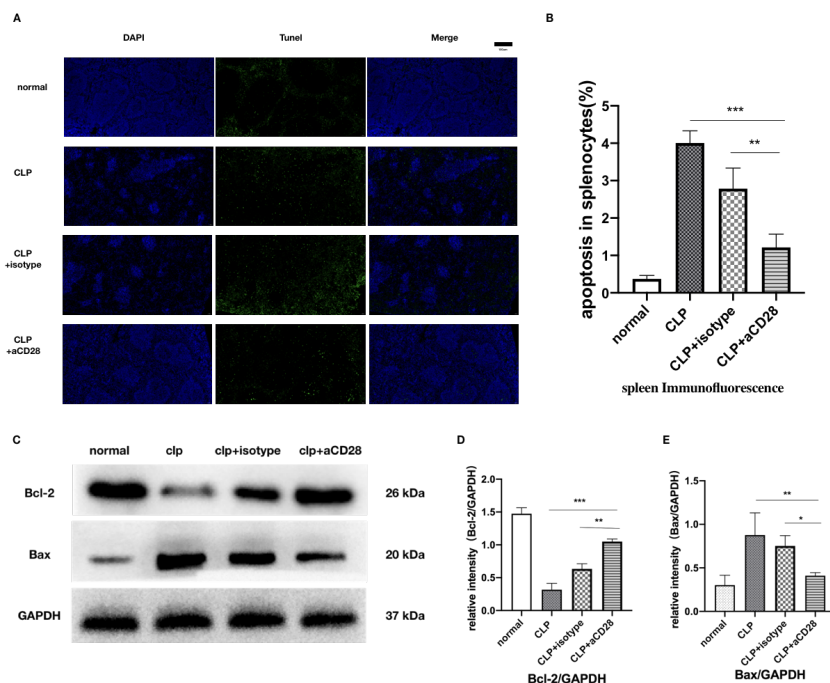


Fig.7 **A**, apoptosis by TUNEL analysis (Original magnification: 200X Scale:100um). Representative confocal fluorescence microscopy images of spleen cells stained with antibodies against TUNEL (green) and DAPI(blue). DAPI, 4',6-diamidino-2-phenylindole. Scale bar, 20 mm.**B**, the percentage of apoptosis were determined average gray value by Image J. **C**, western-blot of apoptosis BCL-2 and Bax in spleen, **D** and **E**, Densitometric analysis of the relevant bands was performed by Image J. The values presented are mean \pm SEM (n=5). *p<0.05, **p<0.01, ***p<0.001, n=5, The data are representative of 3 independent experiments.

Discussion

The body immune system plays an important role in sepsis like double-edged sword, which protects against invading pathogens, while its overreaction can cause body organ injury. During sepsis, the invasion of a large number of pathogens not only causes the body's immune overreaction, but also causes a large number of lymphocytes to consume and cause immune paralysis. As Hotchkiss RS described that the T cells were the band conductor of the orchestra, and all immune components worked in harmony to play the best role[30]. Due to the intertwined complex relationship, Th17, Treg and their balance proportion has been attracted more and more attention, which is essential for immune homeostasis[31]. Although Treg represent less than 10% of circulating CD4+T cells, it can limit the overreaction of anti-infective effector cells, protect surrounding normal tissues from damage, and maintain the body's immune balance [32, 33]. Th17 terminally differentiated cells fulfill opposite Treg's functions; IL-17 can promote the occurrence of inflammation in sepsis[34]. Studies have found that Th17 can appear in lung tissue to help eliminate bacteria in the bacterial infection model, including *Klebsiella pneumoniae* that is resistant to carbapenem[35], which is very important to resistance in sepsis bacterial infection. Th17 cause autoimmunity and inflammation, whereas Treg inhibit these phenomena and maintain immune homeostasis. The two are functionally antagonistic to each other, but also inhibit each other in differentiation, even switch between each other[36]. Thus, unraveling the mechanisms that affect the Th17/Treg balance is critical to understand autoimmunity and tolerance better. Limited data had shown the ratio of Th17/Treg might be related to the clinical severity and prognosis of sepsis [37], [38], [39].

The length of stay in intensive care units (LSICU) is an essential factor for quality assessment and a strong determinant for the total cost of ICU admission, of which septic patients represent a major part[40]. Age

was an independent risk factor of many diseases, and some researches showed that the odds for death in ICU patients with sepsis increased with age with the maximal rate of increase [46]. Age is a factor affecting the length of stay in ICU in patients with sepsis, and there is a positive correlation between age and length of stay in ICU. Clinical scores except age, such as the APACHE II[41], SOFA[42] have been widely used in clinical practice to predict outcome in critically ill patients; the early risk-stratification of these patients and their prognosis, as well as accurate monitoring of clinical treatment effects. Th17/Treg ratio can predict 28-day mortality in sepsis patients with ARDS [43]. There is currently an apparent lack of an accurate biomarker for predicting sepsis patients the length of stay in ICU. In the present study, positive correlations were observed between the Th17/Treg ratio and the length of stay in ICU, indicating that the higher the Th17/Treg ratio, the longer the length of stay in ICU in sepsis patients. The Th17/Treg ratio, APACHE II score, and SOFA score were independent predictors of the length of stay in ICU in sepsis patients, the Th17/Treg ratio in combination with the APACHE II score increased the AUC for predicting the length of stay in ICU. Taken together, our findings strongly suggest that the Th17/Treg ratio can reflect the intensity of the inflammatory response in sepsis, and might be a potential indicator for the length of stay in ICU in sepsis patients.

CD28 is the primary costimulatory molecule for naive CD4+ conventional T cell activation[44], binding to B7 ligands leads to increased duration and magnitude of T cell responses, enhanced survival and glucose metabolism, and acquisition of migratory properties[45]. CD28 activates integrin-mediated adhesion of T cells and promotes actin polymerization. Selective blockade of CD28-mediated T cell costimulation protects rhesus monkeys against acute fatal experimental autoimmune encephalomyelitis(EAE)[46]. In septic patients, there is a strong positive correlation between CD28 expression on CD4+ T cells and Th17/Treg ratio; we speculate that CD28 may be able to change Th17/Treg ratio then short the length of stay in ICU.

The mice sepsis model showed that the clinical score and Th17/Treg ratio of septic mice were the highest on the third day after CLP. After administration of CD28 antibody, the 7-day survival rate of mice increased, the Th17/Treg ratio of spleen cells decreased, and the expression of inflammatory factors in peripheral serum also decreased. Simultaneously, the spleen's length, weight and index were improved, and the histopathological damage were alleviated. These suggested the decrease of apoptosis of spleen cells in mice. It is speculated that CD28 antibody may affect the balance between Th17 and Treg through ROR- γ t and FoxP3, thereby improving the survival rate of sepsis mice.

The function of T cells is related to cell death, which can be divided into accidental cell death (ACD) and regulatory cell death (RCD). The signal cascade reactions involved in regulating cell-related functions are mainly RCD, RCD involving effector molecules, which have unique biochemical characteristics, morphological characteristics and immunological consequences [47]. RCD mode includes apoptosis, necroptosis, pyroptosis, ferroptosis, entotic cell death, and so on.[48]. T cell dysfunction is manifested specifically in increased T cell apoptosis, decreased proliferative capacity and decreased reactivity or unresponsive state[49]. Our data showed that the administration of CD28 antibody to CLP processed mouse could reduce the apoptosis of splenocytes. Western blot in tissues confirmed that the expression of pro-apoptotic protein BCL-2 decreased and the expression of anti-apoptotic protein BAX increased in mouse spleen under the action of CD28 antibody.

Limitations

There were some limitations to this study. First, this is a single-center study and the sample size was not large, thus restricting generalizability. Our data were used to observe the circulating Th17 and Treg changes and the correlation between Th17/Treg ratio and length of ICU stay. In addition, patients were included for sepsis as a variety of risk factors, and our findings need to be confirmed by further studies and more patients. Second, the change in functional immunocompetent cells was only measured at one time point, because we aimed to understand the early functional immune cells influence on clinical outcomes. Many factors affect the length of hospital stay of patients with sepsis, not all of them were observed. Therefore, further research is necessary to include more factors that influence hospital stay length of sepsis patients. Finally, Due to the cost and experimental conditions, we did not purify CD4+T lymphocytes from human

PBMC, nor did we conduct further mechanism verification. CD28 knockout mice were not used in our in vivo study for verification, and the intervention only used CD28 antibody, and CD28 agonist was not used. As for the mechanism of the effect of CD28 antibody on T cell function, we only focused on the splenocytes' apoptosis, but did not further explore other possible mechanisms.

CONCLUSIONS

Th17/Treg ratio can be used as a predictor of ICU stay in sepsis. CD28 functional antibody may have therapeutic significance for sepsis patients and treat septic mice by affecting the imbalance of Th17/Treg ratio and inhibiting T cell apoptosis.

Abbreviations

APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sepsis-related organ failure assessment; AUC: Area under the receiver operating characteristic curve; EDTA: Ethylenediaminetetraacetic acid; GCS: Glasgow Coma Scale; ICU: Intensive care unit; IFN- γ : Interferon- γ ; IL-4: Interleukin-4; IL-8: Interleukin-8; IL-10: Interleukin-10; IL-17A: Interleukin-17A; OR: Odds ratio; PCT: Procalcitonin; ROC: Receiver operating characteristic; WBC: White blood cell; CD3: Cluster of Differentiation 3; CD4: Cluster of Differentiation 4; CD8: Cluster of Differentiation 8; CD28: Cluster of Differentiation 28; CD80: Cluster of Differentiation 80; CD86: Cluster of Differentiation 86; CTLA-4: cytotoxic T-lymphocyte-associated protein 4;

Acknowledgements

Not applicable.

Authors' contributions

Yu Wu performed the mostly experiments, analyzed the data, drafted the manuscript. Dai Li and Jian Xie analyzed the data, and critically revised the manuscript. Han Wang, Yan Meng and Guosheng Wu contributed to the data analysis and interpretation. Xiaoming Deng conceived and designed the study. Xiaojian Wan conceived and designed the experiments, analyzed the data, edited the manuscript. All authors substantially contributed to editing, revising, and finalizing the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by Shanghai Natural Science Foundation (19ZR1456600), National Natural Science Foundation of China (81600955), Shanghai Pujiang Program (2020PJD059) and Hebei Medical Science Research Key Project Plan (ZD20170953)

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was reviewed and approved by the Changhai Hospital Ethics Committee. It was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients or their legal proxy before enrollment. All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, China, 1998).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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