

Up-regulation of Programmed Cell Death 1 Ligand 1 on Neutrophils May Be Involved in Sepsis-induced Immunosuppression

An Animal Study and a Prospective Case-control Study

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ABSTRACT

Background: Recent studies have shown that neutrophils may display an antigen-presenting function and inhibit lymphocyte proliferation by expressing programmed cell death 1 ligand 1 (PD-L1). The current study was performed to investigate the effect of neutrophils and their pathophysiological significance during sepsis.

Methods: Neutrophil PD-L1 expression was determined in both septic mice ($n = 6$) and patients ($n = 41$). Neutrophils from septic mice were subtyped into PD-L1⁻ and PD-L1⁺ populations to determine their phenotypes and functions. Septic neutrophils were cocultured with lymphocytes to observe the effect of septic neutrophils on lymphocyte apoptosis.

Results: The PD-L1 level on neutrophils from septic mice was significantly up-regulated ($21.41 \pm 4.76\%$). This level increased with the progression of sepsis and the migration of neutrophils from the bone marrow to the blood and peritoneal cavity. The percentages of CD11a, CD62L, and C-C chemokine receptor type 2 were lower, whereas the percentages of CD16 and CD64 were higher on PD-L1⁺ neutrophils than on PD-L1⁻ neutrophils. The migratory capacity of PD-L1⁺ neutrophils was compromised. Septic neutrophils induced lymphocyte apoptosis *via* a contact mechanism, and this process could be reversed by anti-PD-L1 antibody. PD-L1 was also up-regulated on neutrophils from patients with severe sepsis ($14.6\% [3.75\%, 42.1\%]$). The levels were negatively correlated with the monocyte human leukocyte antigen-DR level and positively correlated with the severity of septic patients. Neutrophil PD-L1 was a predictor for the prognosis of severe sepsis, with an area of 0.74 under the receiver operating curve.

Conclusions: PD-L1 is up-regulated on neutrophils during sepsis, which may be related to sepsis-induced immunosuppression. (ANESTHESIOLOGY 2015; 122:852-63)

SEPSIS is defined as an infection-induced systemic inflammatory response syndrome, and severe sepsis refers to organ dysfunction caused by infection.¹ Several epidemiological studies have shown that the mortality of severe sepsis is higher than 40%, and sepsis has become the third most common cause of death in the United States.²⁻⁴ It was previously thought that overwhelming inflammatory responses after infection were the main cause of death, whereas antiinflammatory agents such as anti-tumor necrosis factor- α antibody and soluble interleukin-1 receptor showed no beneficial effects against sepsis.^{5,6} Although the advances in medical technologies, including mechanical ventilation and continuous renal replacement therapies, have improved the survival rates of most patients with sepsis-induced lung or kidney failure, uncontrolled primary or secondary infection resulting from sepsis-induced dysregulation of the immune

What We Already Know about This Topic

- It has been shown that neutrophils may display an antigen-presenting function and inhibit lymphocyte proliferation by expressing programmed cell death 1 ligand 1

What This Article Tells Us That Is New

- Programmed cell death 1 ligand 1 is up-regulated on neutrophils during sepsis, which may be associated with sepsis-induced immunosuppression

system is known to be the actual cause of mortality in patients with severe sepsis. This phenomenon is also known as immunosuppression and is represented by the compromised migration and phagocytosis of phagocytes, the dysfunction of antigen-presenting cells (APCs), and the apoptosis and anergy of lymphocytes.^{7,8}

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Programmed cell death 1 ligand 1 (PD-L1), a coinhibitory molecule expressed on APCs, negatively regulates the activation of T-cell receptors and mediates lymphocyte apoptosis.^{9,10} Our previous studies demonstrated that PD-L1 blockade using the specific neutralizing antibody improved the bacterial clearance and prognosis of septic mice and that anti-PD-L1 antibody reversed sepsis-induced lymphocyte apoptosis and the inability of monocytes to secrete cytokines *in vitro*.^{11,12}

Over the last decade, PD-L1 was identified on the surface of neutrophils. A microarray analysis by Malcolm *et al.*¹³ in 2003 showed that PD-L1 (also called B7-H1) messenger RNA was up-regulated on lipopolysaccharide-stimulated neutrophils. A subsequent study reported that neutrophils could express PD-L1 upon the stimulation of interferon- γ and granulocyte-macrophage colony-stimulating factor.¹⁴ Neutrophils were even identified as the main origin of PD-L1 in the blood of patients with active tuberculosis.¹⁵ An *in vitro* study suggested that neutrophils could inhibit lymphocyte-mediated inflammation by expressing PD-L1.¹⁶ A very recent study showed that neutrophil PD-L1 was elevated in septic mice, which was significantly correlated with a poor outcome of sepsis.¹⁷

More studies have shown that neutrophils are involved in the adaptive immune system as APCs.^{18,19} The direct contact of neutrophils with lymphocytes might inhibit lymphocyte proliferation *via* macrophage-1 antigen.²⁰ This inhibitory effect of neutrophils against lymphocytes would be of great importance because they are the most abundant leukocytes in peripheral blood. Although the significance of neutrophil dysfunction has been well defined, the role of PD-L1 expression on neutrophils during sepsis remains to be clarified.

We hypothesized that neutrophils might also express PD-L1 during sepsis because of the complex changes of cytokines. PD-L1 expression on neutrophils might also be involved in the immunosuppression induced by sepsis because PD-L1 might induce the apoptosis of immune cells expressing PD-L1. The aim of the current study was to determine the expression level of PD-L1 on neutrophils in both septic mice and patients. Neutrophils expressing PD-L1 were then distinguished from PD-L1⁻ populations by analyzing their phenotype and migration ability. Whether neutrophils derived from septic mice could induce lymphocyte apoptosis by PD-L1 was also investigated using a transwell system and anti-PD-L1 antibody. Finally, the clinical significance of neutrophil PD-L1 was analyzed to assess whether it correlated with immunosuppression, disease severity, and prognosis.

Materials and Methods

Mouse and Cecal Ligation Puncture Model

The study protocol pertaining to the animal experiments was approved by the Animal Care and Use Committee of Changhai Hospital (Shanghai, China). Adult C57BL/6 mice aged 6 to 8 weeks were purchased from the Experimental Animal

Center of the Second Military Medical University (Shanghai, China). All of the animals were acclimated under a 12-h light/12-h dark cycle for 1 week before the experiments. The mice were randomly subjected to cecal ligation puncture (CLP) surgery or sham operation through a randomization table. For each comparison of animal experiments, six mice were allocated to each group based on our previous experience.^{11,12} The CLP surgery was performed as previously described.²¹ Briefly, after sevoflurane-inhalation anesthesia, the cecum was ligated below the ileocecal valve with a 1-0 Prolene thread. The ligated cecum was then punctured with a 22-gauge needle to induce polymicrobial peritonitis. In the sham-operated mice, the cecum was exposed using a similar procedure but without ligation and puncture. After surgery, mice were resuscitated with 1 ml of sterile physiologic saline. All of the mice were given free access to food and water after recovery from anesthesia.

Reagents, Antibodies, and Flow Cytometry

Cells were cultured in Roswell Park Memorial Institute 1640 (Gibco, Brooklyn, NY) supplemented with 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria), 100 U/ml penicillin (Gibco), 100 ng/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco). Neutrophils were isolated using Percoll solution (Pharmacia, Stockholm, Sweden) and resuspended in Hanks Balanced Salt Solution (LONZA, Basel, Switzerland). N-formylmethionyl-leucyl-phenylalanine (Sigma-Aldrich, St. Louis, MO) and EDTA (Sinopharm Chemical Reagent, Shanghai, China) were used in the chemotaxis assay, and the transwell microchamber was purchased from Millipore (Bedford, MA). Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA), including mouse Gr-1 FITC, PD-L1 PE, PD-L1 APC, CD11a APC, CD11b APC, CD16 APC, CD18 PE, CD54 APC, CD62L PE, CD64 PE, C-C chemokine receptor type 2 (CCR2) PE, MHC-II APC, CD40 PE, CD80 PE, CD86 APC, CD3 APC, and IgG2b κ Isotype APC; and human CD3 PE, CD14 APC, CD15 FITC, PD-L1 PE, programmed death receptor 1 (PD-1) APC, human leukocyte antigen DR (HLA-DR) PE-Cy5, and IgG1 K Isotype PE. The apoptosis assay kit for flow cytometry and the PD-L1 neutralization antibody as well as the IgG2a K Isotype antibody were also purchased from eBioscience. Cells were analyzed in a FACSCalibur using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Detection of Membrane Molecules on Neutrophils

PD-L1, major histocompatibility complex (MHC) class II molecule, CD40, CD80, and CD86 on mouse Gr-1⁺ neutrophils were detected by flow cytometry. Blood samples were harvested at 12, 18, and 24 h after surgery to observe dynamic changes of the PD-L1 level on blood neutrophils ($n = 6$ for each time point). PD-L1 on neutrophils from the bone marrow, blood, and peritoneal cavity was also detected at 24 h after surgery to better understand the expression of

neutrophil PD-L1 from different locations ($n = 6$ for each location). MHC class II molecule, CD40, CD80, and CD86 on neutrophils were detected to assess whether the neutrophils had any stable contact effect on lymphocytes at 0, 12, 18, and 24 h after surgery ($n = 6$ for each time point). The results are presented as a percentage of positive cells. All of the flow cytometry analyses were performed independently by an investigator who was blinded to the sample grouping.

Identification of PD-L1⁺ Neutrophils

According to the PD-L1 expression status, neutrophils were classified as Gr-1⁺PD-L1⁺ and Gr-1⁺PD-L1⁻ populations using the CellQuest software (Becton Dickinson) after being standardized with anti-Gr-1 and anti-PD-L1 single staining. The two populations were then gated to detect a third molecule reflecting the neutrophil phenotype, including CD11a, CD11b, CD16, CD18, CD54, CD62L, CD64, and CCR2.

Purification and Transwell Assay of Neutrophils

Blood neutrophils were purified with a Percoll solution using a 52%, 69%, and 78% gradient, as previously described, and resuspended in Hanks Balanced Salt Solution.²² The chemotaxis assay was performed in a 24-well transwell microchamber using a polycarbonate membrane with 5 μ m pores. The neutrophils (1×10^6 cells/ml) were added to the upper chamber, and N-formylmethionyl-leucyl-phenylalanine (1×10^{-7} mmol/l) was used as the chemotactic agent. Two hours later, 50 μ l of EDTA (70 mM) was added to the lower chamber to detach the neutrophils from the membrane. The neutrophils were analyzed by flow cytometry to detect the percentage of neutrophils that migrated into the lower chamber.

Coculture of Neutrophils and Spleen Mononuclear Cells

Mononuclear cells were isolated from the spleen of normal mice with a 60% Percoll solution. First, neutrophils from the blood of septic mice or sham mice were cocultured with normal spleen mononuclear cells, in both direct and indirect contact (separated by the transwell chamber), at a 1:1 ratio (1×10^6 cells/ml) in Roswell Park Memorial Institute 1640 solution. Second, neutrophils from the blood of septic mice were directly cocultured with normal spleen mononuclear cells and treated with anti-PD-L1 antibody (10 μ g/ml) or IgG2a K Isotype antibody (10 μ g/ml). Single spleen mononuclear cells that were treated with anti-PD-L1 antibody or isotype antibody served as the control. Lymphocyte apoptosis in spleen cells were induced by tumor necrosis factor- α (10 ng/ml). The induction of apoptosis was accomplished in an incubator (37°C, 5% CO₂) for 24 h. CD3⁺ lymphocyte apoptosis was determined by annexin-V and propidium iodide staining. Cells positive for annexin-V and negative for propidium iodide were considered apoptotic.

Clinical Settings

The following clinical study was a prospective case-control study. The subjects were included from June 2011 to January

2013 and consisted of patients with severe sepsis, sepsis after percutaneous nephrolithotomy because of an infectious kidney stone, pancreatic cancer, and healthy volunteers. All of the septic patients were recruited from the Intensive Care Unit (ICU) of Changhai Hospital, and the cancer patients were recruited from the Department of General Surgery in the same hospital. Healthy volunteers were randomly recruited from healthy patients who underwent routine physical examinations. Sepsis was defined according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference.²³ Generally, sepsis was diagnosed by an identifiable or suspected infection site and evidence of Systemic Inflammatory Response Syndrome manifested by at least two of the following criteria: (1) a body temperature higher than 38°C or lower than 36°C; (2) a heart rate higher than 90 beats/min; (3) a respiratory rate higher than 20 breaths/min; or (4) a leukocyte count higher than 12,000 cells/mm³ or lower than 4,000 cells/mm³. Severe sepsis was diagnosed when the septic patient suffered from at least one organ dysfunction within 24 h after inclusion. This study was approved by the Committee on Ethics of Biomedicine Research of the Second Military Medical University. Informed consent was obtained from all of the patients or their surrogates before recruitment. This part of the study was registered with Clinicaltrial.gov under NCT01976884.

Data collection included general parameters such as gender, age, primary diagnosis, and infection site, as well as severity score, including the Acute Physiology and Chronic Health Evaluation II, Sequential Organ Failure Assessment, and Multiple Organ Dysfunction Syndrome scores, the length of stay in the ICU, ventilation duration, and 28-day mortality. Monocyte HLA-DR and lymphocyte PD-1 were detected by flow cytometry to assess the correlation between neutrophil PD-L1 expression and immunosuppressive status.

Statistical Analysis

All of the data were analyzed in SPSS 16.0 (SPSS, Chicago, IL). Continuous data in a normal distribution were expressed as the mean \pm SD, and those in a nonnormal distribution were expressed as the mean (minimum, maximum). Comparisons between two groups of continuous data were analyzed using Student *t* test, and comparisons among three or more groups were analyzed using an ANOVA, followed by the Student–Newman–Keuls test. Nonnormal distribution was compared using the Mann–Whitney *U* test and Kruskal–Wallis *H* test for two-group and multiple-group comparisons, respectively. The Nemenyi test was conducted for *post hoc* adjustments after the Kruskal–Wallis *H* test. Correlation analysis was performed using linear regression analysis. The patients with severe sepsis were classified into two groups based on the median value of neutrophil PD-L1 to evaluate the differences in disease severity, the duration of mechanical ventilation, and the length of ICU stay. Receiver operating curve analysis was performed to evaluate the prognostic value of neutrophil PD-L1, monocyte HLA-DR, and lymphocyte PD-1. The

sample size was not calculated in this study because this is the first study to report the PD-L1 expression levels on neutrophils from patients with sepsis, and no evidence could be referenced for statistical power analysis. Instead, the sample size was determined by our previous experience. A P value less than 0.05 was considered statistically significant.

Results

Up-regulation of PD-L1 on Neutrophils in CLP Mice

Neutrophil PD-L1 was significantly up-regulated in CLP mice 12 h after surgery ($4.08 \pm 0.65\%$), whereas it was negligible in sham-operated mice. Neutrophil PD-L1 was higher at 18 h than at 12 h and peaked within 24 h after CLP surgery, with a mean level of $21.41 \pm 4.76\%$, which was significantly higher than that observed in Sham mice ($P < 0.001$) (fig. 1A; for detailed statistical data, see fig. 1A, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>). Inflammation mobilizes neutrophils into circulation, which then migrate to the inflammatory sites such as the peritoneal cavity. Interestingly, negligible PD-L1 was detected on neutrophils from the bone marrow of CLP mice 24 h after surgery. The neutrophil PD-L1 levels in the peritoneal cavity were as high as $55.85 \pm 11.4\%$, which was significantly higher than that in the blood and bone marrow 24 h after

surgery ($P < 0.001$) (fig. 1B; for detailed statistical data, see fig. 1B, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>).

Phenotype and Migration Ability of PD-L1⁺ Neutrophils

Because the percentage of PD-L1-positive neutrophils was significantly elevated, we gated neutrophils into PD-L1⁺ and PD-L1⁻ subtypes in flow cytometry (fig. 2) and compared several surface molecules between the subtypes. CD11b, CD18, and CD54 were fully expressed in almost all of the cells (approximately 99%) in the two populations. The CD11a and CD62L levels were lower, and the CD16, CD64, and CCR2 levels were higher on PD-L1⁻ neutrophils of CLP mice compared with Sham mice, whereas the CD11a, CD62L, and CCR2 levels were lower and the CD16 and CD64 levels were higher on PD-L1⁺ neutrophils of CLP mice compared with PD-L1⁻ neutrophils. The change in the mean fluorescence intensity of these surface molecules was similar to the percentages, with the exception of CD18, CD54, and CD62L. PD-L1⁺ neutrophils had a higher mean fluorescence intensity of CD18 and CD54 than did PD-L1⁻ neutrophils, whereas CD62L was similar on the two subsets of neutrophils (fig. 3; for detailed statistical data, see fig. 2, Supplemental Digital Content 1, <http://links.lww.com/>

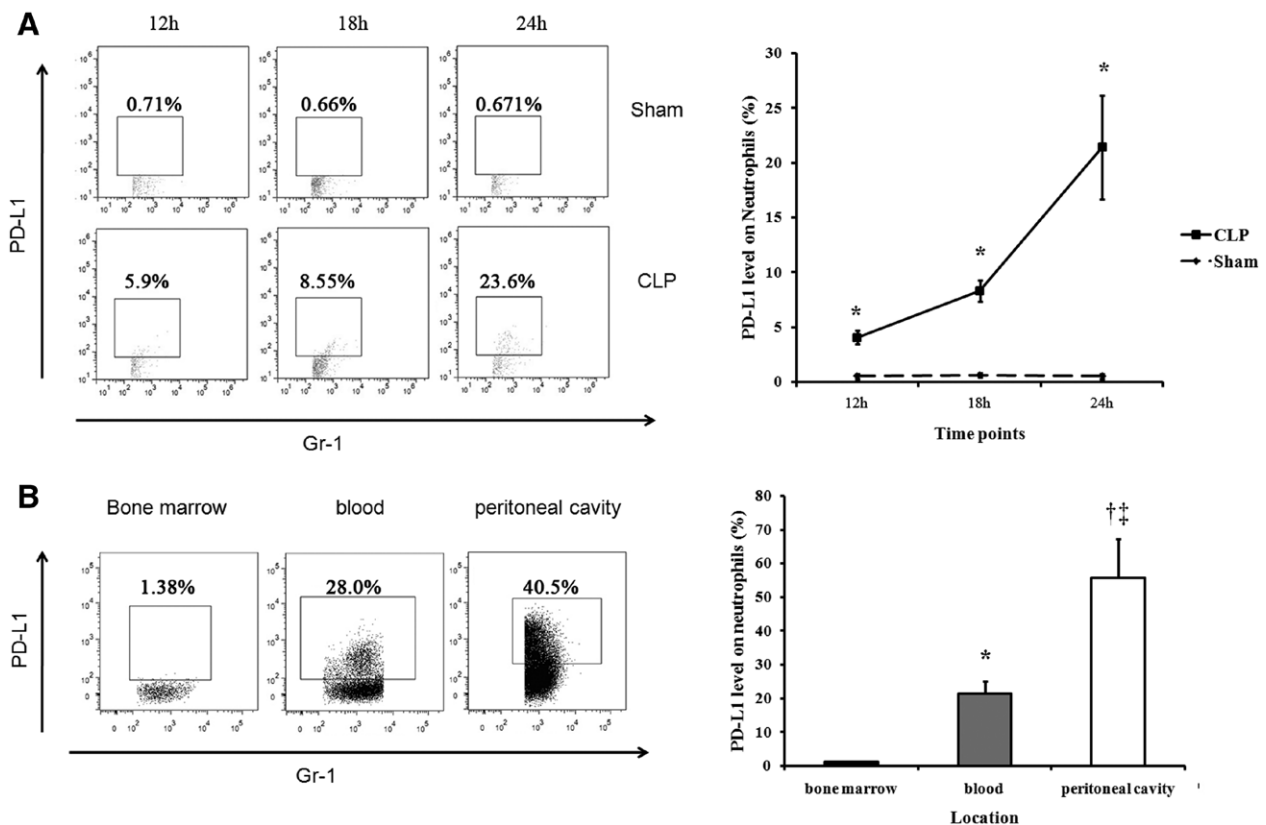


Fig. 1. Programmed death receptor 1 ligand 1 (PD-L1) expression on neutrophils at different time points after cecal ligation puncture (CLP) surgery and in different locations at 24 h after surgery. (A) PD-L1 level on neutrophils at 12, 18, and 24 h. * $P < 0.05$ compared with sham group by Student t test. (B) PD-L1 level on neutrophils from bone marrow, blood, and peritoneal cavity. *, $\dagger P < 0.05$, compared with bone marrow; $\ddagger P < 0.05$, compared with blood by ANOVA.

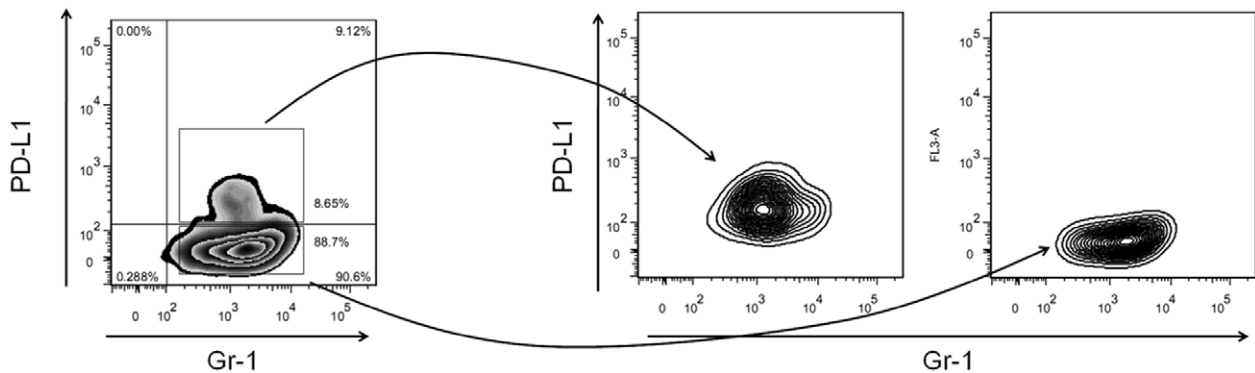


Fig. 2. Identification of programmed death receptor 1 ligand 1 (PD-L1)⁺ neutrophils by flow cytometry.

ALN/B113). The percentage of neutrophils migrating into the lower chamber was reduced in CLP mice compared with that in sham-operated mice ($P = 0.003$), and fewer PD-L1⁺ neutrophils migrated into the lower chamber compared with PD-L1⁻ neutrophils ($P = 0.013$) (fig. 4; for detailed statistical data, see fig. 3, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>).

APC Characteristics of Neutrophils during Sepsis

Because PD-L1 is an inhibitory molecule that negatively regulates the antigen-presenting process, we detected the antigen-processing-related molecules on neutrophils, including MHC class II molecules, CD40, CD80, and CD86, to investigate whether neutrophils can act as APCs during sepsis. The expression level of these molecules on neutrophils was within 5% in normal mice (0 h), increased at 18 h after CLP surgery, and peaked at 24 h after surgery (fig. 5; for detailed statistical data, see fig. 4, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>).

The Effect of Neutrophil PD-L1 on Lymphocyte Apoptosis in CLP Mice

Several studies have reported that neutrophils might inhibit lymphocyte activity in certain circumstances.^{16,20} We therefore cocultured normal spleen mononuclear cells with neutrophils from the blood of CLP or sham-operated mice in a direct-contact or indirect manner to observe whether neutrophils could induce lymphocyte apoptosis *via* a direct-contact mechanism. An indirect coculture with neutrophils from CLP mice using a transwell chamber resulted in a slight increase in the number of apoptotic lymphocytes compared with a direct coculture with neutrophils from sham-operated mice ($P = 0.015$). Conversely, a direct coculture with neutrophils from CLP mice increased the apoptotic level of CD3⁺ lymphocytes significantly compared with an indirect coculture with neutrophils from CLP mice or a direct coculture with neutrophils from sham-operated mice ($P < 0.001$) (fig. 6A; for detailed statistical data, see fig. 5A, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>). Then, anti-PD-L1 antibody was added to the direct coculture system to investigate whether this direct-contact

mechanism was mediated by PD-L1. Anti-PD-L1 antibody, but not the isotype antibody, almost completely reversed the lymphocyte apoptosis induced by neutrophils from CLP mice ($P < 0.001$). We previously determined that the anti-PD-L1 antibody inhibited tumor necrosis factor- α -induced lymphocyte apoptosis *in vitro*,^{11,12} so we used anti-PD-L1 antibody-treated mononuclear cells without neutrophil coculture as a control in this study. In the absence of neutrophils, lymphocyte apoptosis was slightly reduced by anti-PD-L1 antibody compared with the isotype antibody ($P = 0.033$) (fig. 6B; for detailed statistical data, see fig. 5B, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>). The results suggest that the neutrophils from CLP mice induced lymphocyte apoptosis in a direct-contact mechanism *via* PD-L1 mediation.

Expression of Neutrophil PD-L1 in Patients with Sepsis

A total of 41 patients with severe sepsis, 10 patients with sepsis after percutaneous nephrolithotomy, 10 cancer patients, and 10 healthy volunteers were recruited in this study. The general characteristics of the patients with severe sepsis are listed in table 1. Briefly, the primary diagnosis of the patients with severe sepsis included intestinal obstruction, digestive tract perforation, intestinal fistula, pancreatic fistula, pneumonia, mesenteric artery thrombosis, and catheter-related bloodstream infection. Twenty patients died within 28 days after recruitment. Similar to CLP mice, the PD-L1 level on neutrophils was significantly elevated in patients with severe sepsis, with a median level of 14.6% (3.74%, 42.1%). All of the patients with sepsis after percutaneous nephrolithotomy because of infectious kidney stones had a short disease duration and recovered quickly; therefore, immunosuppression seldom occurred in this group. The median level of PD-L1 in patients with kidney stones was $3.45 \pm 1.58\%$, which was significantly lower than that in patients with severe sepsis ($P = 0.009$). In cancer patients and volunteers, the PD-L1 level on neutrophils was below 1%, which was much lower than that in patients with severe sepsis ($P < 0.001$ for cancer patients and volunteers) (fig. 7; for detailed statistical data, see fig. 6, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>).

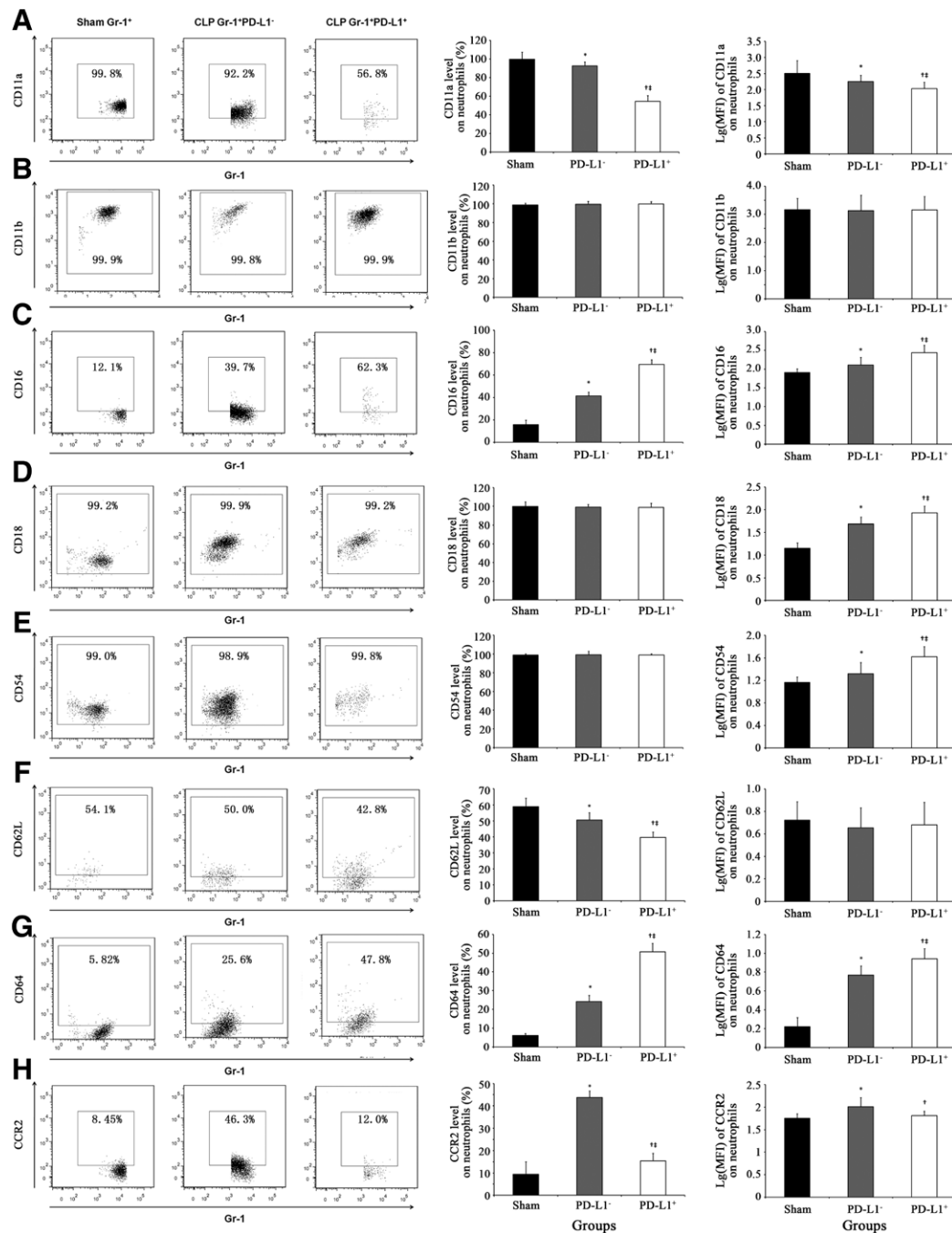


Fig. 3. Comparison of CD11a (A), CD11b (B), CD16 (C), CD18 (D), CD54 (E), CD62L (F), CD64 (G), and C-C chemokine receptor type 2 (CCR2) (H) between neutrophils from sham-operated mice and programmed death receptor 1 ligand 1 (PD-L1)⁺ and PD-L1⁻ neutrophils from cecal ligation puncture (CLP) mice. *, † $P < 0.05$, compared with neutrophils from sham-operated group; † $P < 0.05$, compared with PD-L1⁻ neutrophils by ANOVA.

Correlations between Neutrophil PD-L1 and Immunosuppression, Disease Severity, and Prognosis in Patients with Severe Sepsis

Correlations between neutrophil PD-L1 and other immunosuppressive molecules were analyzed, including monocyte HLA-DR and lymphocyte PD-1, both of which are markers of immunosuppression. Interestingly, neutrophil PD-L1 was linearly correlated with monocyte HLA-DR ($P = 0.010$),

but not lymphocyte PD-1 ($P = 0.139$), which was not correlated with monocyte HLA-DR ($P = 0.255$) (fig. 8A; for detailed statistical data, see fig. 7A–C, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>). Neutrophil PD-L1 was also positively correlated with the severity scores, including Acute Physiology and Chronic Health Evaluation II ($P = 0.021$), Sequential Organ Failure Assessment ($P < 0.001$), and Multiple Organ Dysfunction Syndrome

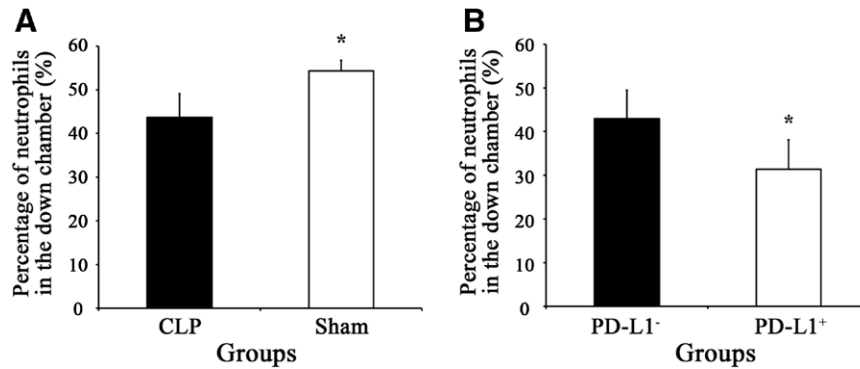


Fig. 4. Migratory ability of neutrophils isolated from sham-operated mice and programmed death receptor 1 ligand 1 (PD-L1)⁺ and PD-L1⁻ neutrophils from cecal ligation puncture (CLP) mice. (A) Comparison between neutrophils from CLP mice and sham-operated mice. (B) Comparison between PD-L1⁺ and PD-L1⁻ neutrophils from CLP mice. * $P < 0.05$ by Student t test.

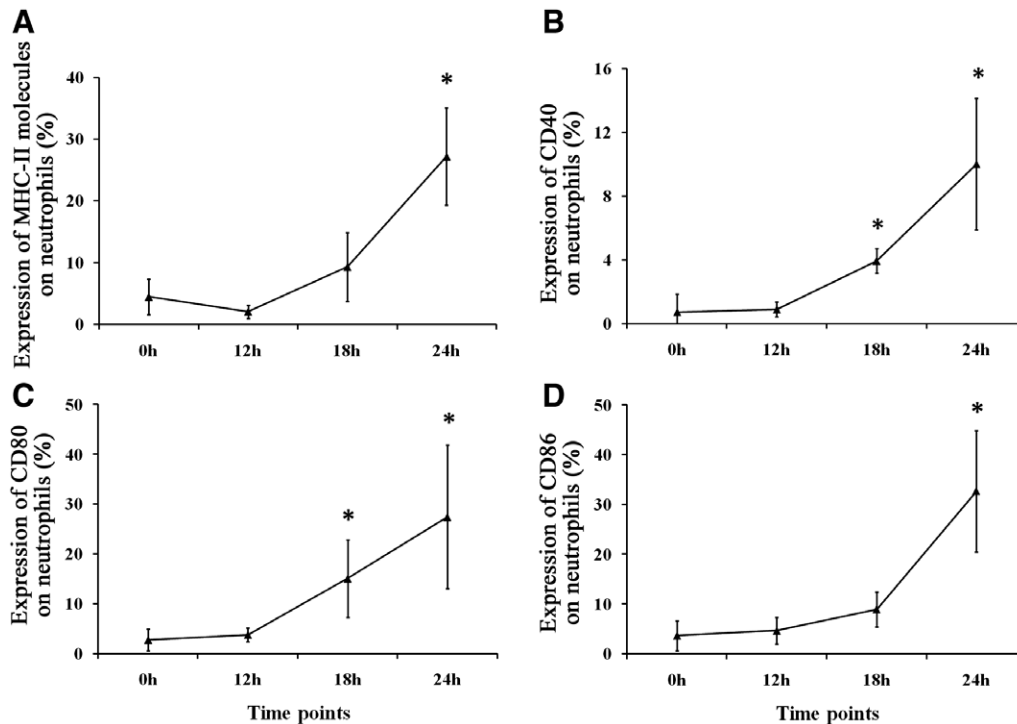


Fig. 5. Expression of major histocompatibility complex (MHC) class II molecule (A), CD40 (B), CD80 (C), and CD86 (D) on neutrophils isolated from cecal ligation puncture mice. * $P < 0.05$ by Student t test, compared with 0h.

($P < 0.001$) (fig. 8B; for detailed statistical data, see fig. 7D–F, Supplemental Digital Content 1, <http://links.lww.com/ALN/B113>). Because the median level of neutrophil PD-L1 in patients with severe sepsis was 14.6%, we selected 15% as a cutoff point to compare the mechanical ventilation and length of stay in the ICU. Patients with a neutrophil PD-L1 level greater than 15% had a much higher degree of sepsis, as demonstrated by Acute Physiology and Chronic Health Evaluation II ($P = 0.002$), Sequential Organ Failure Assessment ($P = 0.001$), and Multiple Organ Dysfunction Syndrome ($P < 0.001$), accompanied by a significantly longer mechanical ventilation ($P = 0.033$) and a slightly prolonged length of ICU stay ($P = 0.06$) (fig. 9; for detailed statistical data, see fig. 8, Supplemental Digital Content 1, [ALN/B113\). To better understand the correlation of neutrophil PD-L1 with the progression of severe sepsis, we evaluated the prognostic value of neutrophil PD-L1, monocyte HLA-DR, and lymphocyte PD-1. The AUCs of neutrophil PD-L1, monocyte HLA-DR, and lymphocyte PD-1 were 0.74, 0.57, and 0.72 in predicting the prognosis of severe sepsis, respectively \(fig. 10\).](http://links.lww.com/</p>
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Discussion

The current study demonstrated that PD-L1 was up-regulated on neutrophils from CLP mice in a time- and location-dependent manner. Neutrophils could be classified into PD-L1⁺ and PD-L1⁻ subtypes. PD-L1⁺ neutrophils were characterized with a specific phenotype (low CD11a,

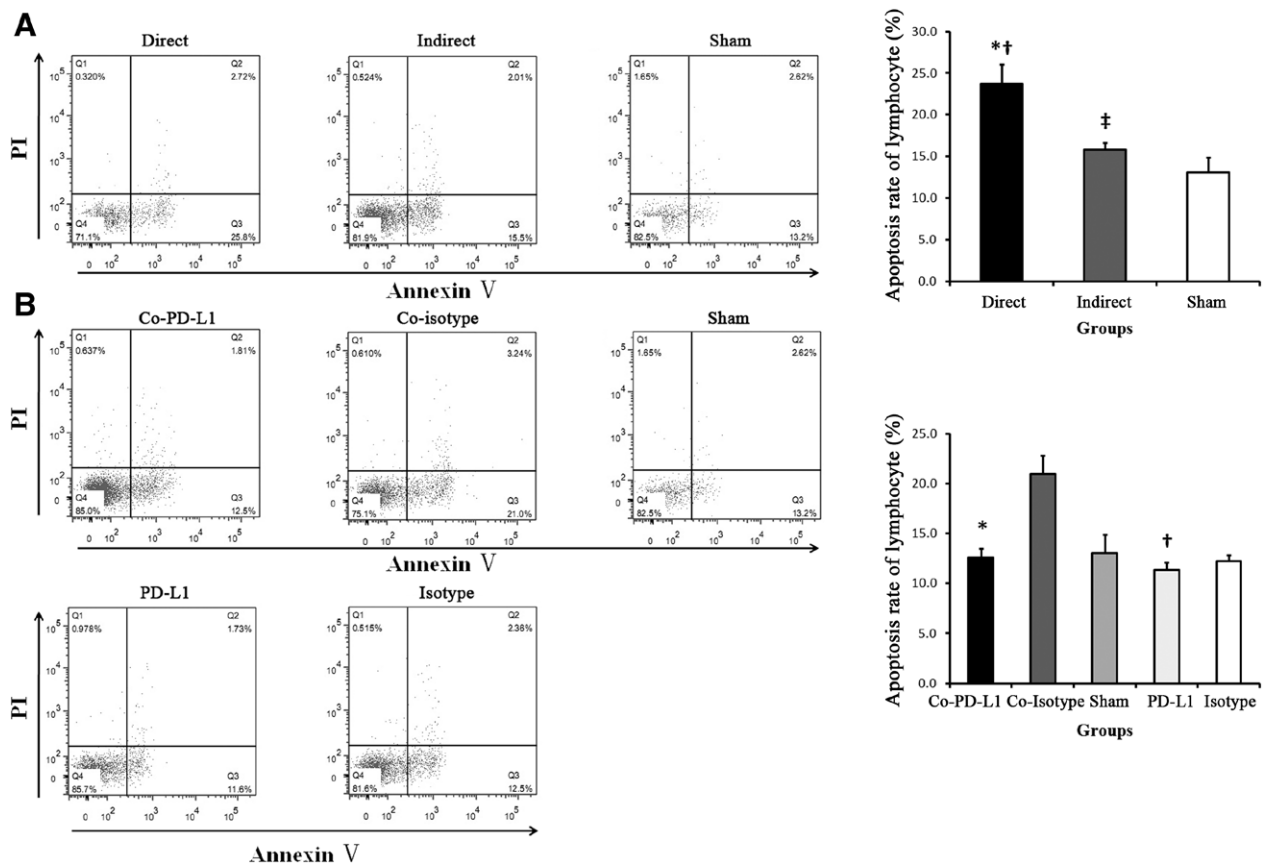


Fig. 6. Influence of programmed death receptor 1 ligand 1 (PD-L1) on neutrophils from cecal ligation puncture (CLP) mice on lymphocyte apoptosis. (A) Direct or indirect coculture of spleen mononuclear cells with neutrophils from CLP mice or sham-operated mice. Indirect coculture was mediated with a transwell chamber. *, † $P < 0.05$ by ANOVA, compared with sham group; ‡ $P < 0.05$ by ANOVA, compared with indirect group. (B) PD-L1 blockade reverse lymphocyte apoptosis induced by neutrophils from CLP mice. Co-PD-L1, spleen mononuclear cells cocultured of neutrophils from CLP mice plus anti-PD-L1 antibody; coisotype, spleen mononuclear cells cocultured of neutrophils from CLP mice plus isotype antibody; sham, spleen mononuclear cells cocultured of neutrophils from sham-operated mice; PD-L1, spleen mononuclear cells plus anti-PD-L1 antibody; isotype, spleen mononuclear cells plus isotype antibody. * $P < 0.05$ by ANOVA, compared with coisotype group; † $P < 0.05$ by ANOVA, compared with isotype group. PI = propidium iodide.

CD62L, and CCR2 levels, and high CD16 and CD64 levels) and compromised the migratory capacity. Neutrophils could induce lymphocyte apoptosis *via* a direct-contact mechanism mediated by PD-L1. Neutrophil PD-L1 was associated with immunosuppression and the severity score of patients with severe sepsis. The neutrophil PD-L1 level was a predictor for the prognosis of severe sepsis.

Several studies have demonstrated that neutrophils express PD-L1 in certain conditions, such as interferon- γ and granulocyte-macrophage colony-stimulating factor stimulation, and during active tuberculosis.^{13–16} Our study suggested that PD-L1 could be considered a subset marker of neutrophils from septic mice. The percentages of CD11a, CD62L, and CCR2 were down-regulated, whereas those of CD16 and CD64 were up-regulated on PD-L1⁺ neutrophils. Mean fluorescence intensities showed that CD18 and CD54 were also up-regulated on PD-L1⁺ neutrophils. CD11a is a surface molecule highly expressed during the early phase of neutrophil development.²⁴ Neutrophil CD11a was considered the

main cause of meningitis-arthritis in dogs because it determined the chemotaxis of neutrophils.²⁵ Low CD11a on PD-L1⁺ neutrophils might be associated with its compromised migratory ability. Similarly, CCR2 was also an important surface molecule involved in neutrophil chemotaxis. Although CCR2 expression was essential for the tissue toxicity of neutrophils during sepsis,²⁶ low CCR2 might be related to a reduced chemotaxis to the infection site. Because neutrophil CD64 was reported to be a potential biomarker for sepsis,^{27–29} high CD64 on PD-L1⁺ neutrophils might reflect possibility that PD-L1⁺ neutrophils are also a marker of infection. High CD16 and low CD62L were reported to be the characters of a special neutrophil subset with a phenotype of CD11c^{bright}/CD62L^{dim}/CD11b^{bright}/CD16^{bright}.²⁰ This subset of neutrophils was highly developed with multisegmented nuclei and exhibited an inhibitory effect against T-cell proliferation. Similarly, high CD16 and low CD62L were present on PD-L1⁺ neutrophils isolated from septic mice, suggesting that PD-L1⁺ neutrophils might also be a multisegmented and

Table 1. Patient Characteristics

Items	Value
Gender (male/female)	29/12
Age	68.9±9.6
Primary diagnosis	
Intestinal obstruction	13
Gastrointestinal tract perforation	8
Intestinal fistula	5
Pancreatic fistula	4
Pneumonia	7
Mesenteric artery thrombosis	2
Catheter-related bloodstream infection	2
Infective site	
Peritoneal cavity	26
Respiratory system	7
Bloodstream infection	2
Multisite infection	6
Severity score	
APACHE II	15.4±5.6
SOFA	6.1±3.4
MODS	4.4±2.9
Outcome (survived/dead)	21/20

APACHE II = Acute Physiology and Chronic Health Evaluation II; MODS = Multiple Organ Dysfunction Syndrome; SOFA = Sequential Organ Failure Assessment.

aged subtype. In addition, our chemotaxis assay demonstrated that PD-L1⁺ neutrophils exhibited compromised migration. Therefore, PD-L1⁺ neutrophils could be considered a special inhibitory population in the development of sepsis. Different PD-L1 levels on neutrophils from the bone marrow, blood, and peritoneal cavity suggest that more mature neutrophils may display a stronger inhibitory effect. The aged neutrophils may transform into inhibitory APCs rather than guard cells in the innate immune system during sepsis.

PD-L1 expression on neutrophils suggested that neutrophils share some aspects with APCs, enabling neutrophil PD-L1 to transmit an inhibitory signal to induce lymphocyte apoptosis. Increasing evidence has shown that neutrophils can act as APCs when they are costimulated with MHC-specific antigen or in an acidic environment.^{30,31} The current study found that neutrophils expressed MHC class II molecule, CD40, CD80, and CD86 during sepsis. Thus, neutrophils might show an antigen-presenting effect during sepsis, but the high level of PD-L1 determined its inhibitory effect on the adaptive immune system. Our *in vitro* results provide evidence that neutrophils from septic mice can directly induce lymphocyte apoptosis in the absence of MHC class II molecule-specific antigen *via* PD-L1. The clinical data further support the speculation that neutrophils may be inhibitory during severe sepsis because neutrophil PD-L1 was correlated with monocyte HLA-DR, which is the gold standard for the diagnosis of sepsis-induced immunosuppression. Neutrophil PD-L1 was also related to the severity score, prolonged ventilation duration, and mortality. These results may suggest a new mechanism underlying sepsis-induced immunosuppression *via* neutrophil mediation.

Finally, the current study demonstrated that neutrophil PD-L1 might be a potential diagnostic biomarker for severe sepsis and sepsis-induced immunosuppression as well as a predictor of mortality. Biomarkers for immunosuppression are critical for patients with severe sepsis because they may provide evidence to support the administration of immune-stimulating therapy. Meisel *et al.*³² used monocyte HLA-DR as a marker for granulocyte-macrophage colony-stimulating factor treatment and found that it was beneficial to restore monocytic immunocompetence. Although hundreds of biomarkers for sepsis have been identified and developed, only monocyte HLA-DR is considered an acceptable

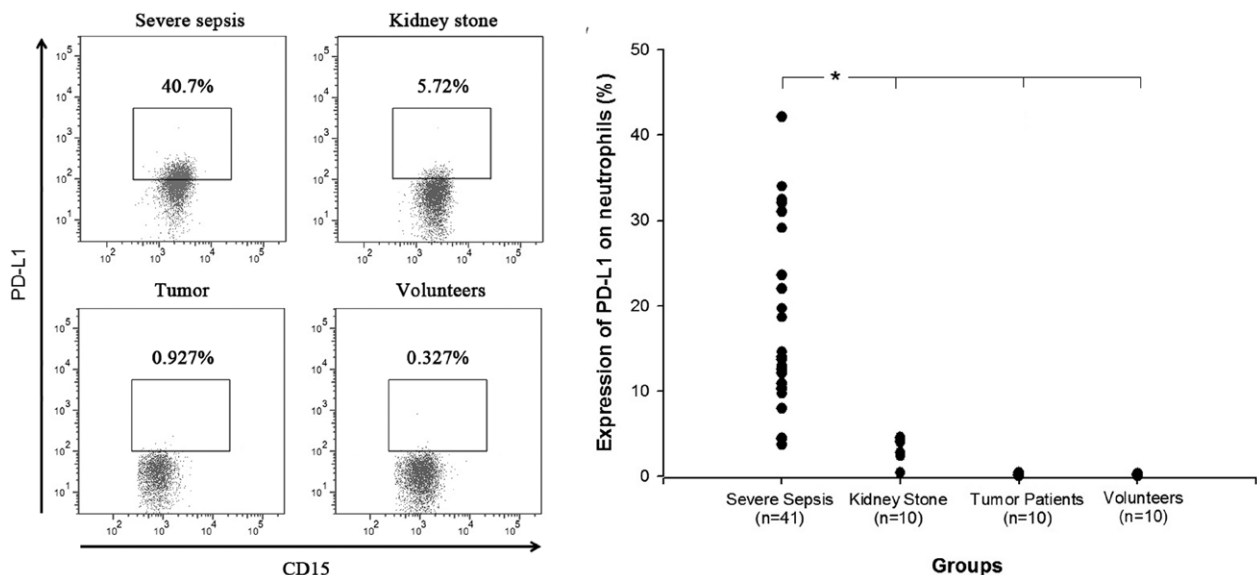


Fig. 7. Programmed death receptor 1 ligand 1 (PD-L1) expression on neutrophils from patients with severe sepsis, sepsis after percutaneous nephrolithotomy for infectious kidney stone, tumor, and healthy volunteers. **P* < 0.05 by Kruskal–Wallis *H* test.

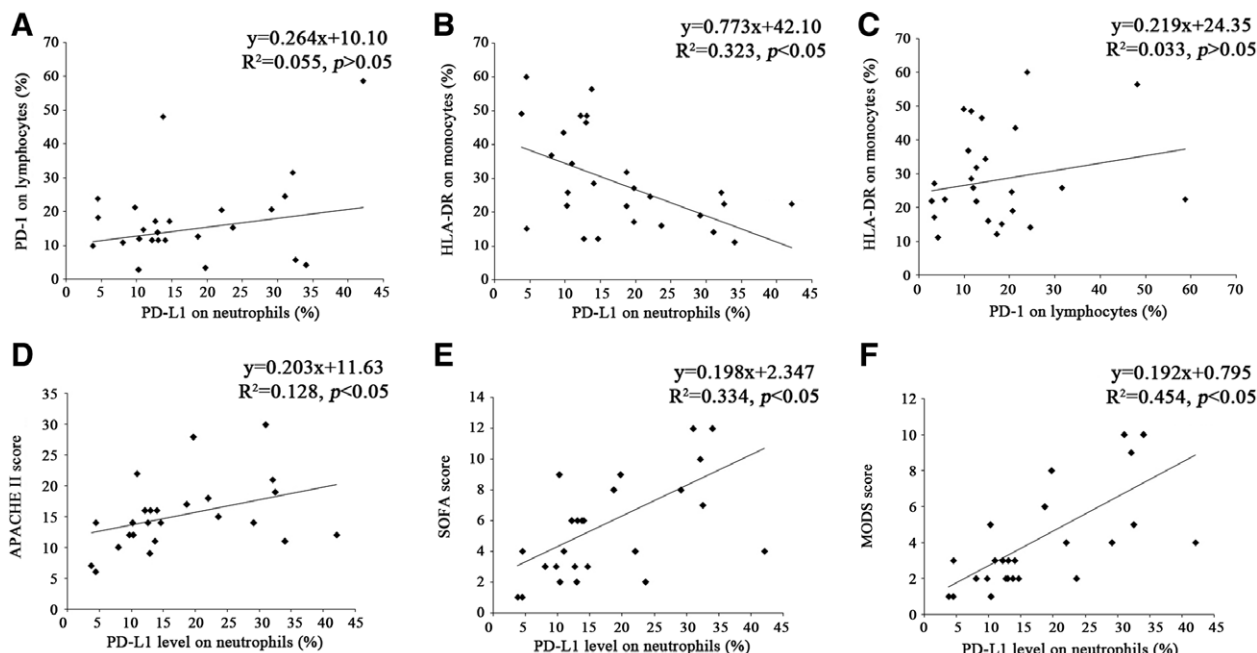


Fig. 8. The correlation of neutrophil programmed death receptor 1 ligand 1 (PD-L1) with marker of immunosuppression. (A) Relationship between lymphocyte programmed death receptor 1 (PD-1) and neutrophil PD-L1; (B) relationship between monocyte human leukocyte antigen DR (HLA-DR) and neutrophil PD-L1; (C) relationship between lymphocyte PD-1 and monocyte HLA-DR and disease severity (correlation of neutrophil PD-L1 with scores of Acute Physiology and Chronic Health Evaluation [APACHE] II [D], Sequential Organ Failure Assessment [SOFA] [E], and Multiple Organ Dysfunction Syndrome [MODS] [F] by linear regression analysis.

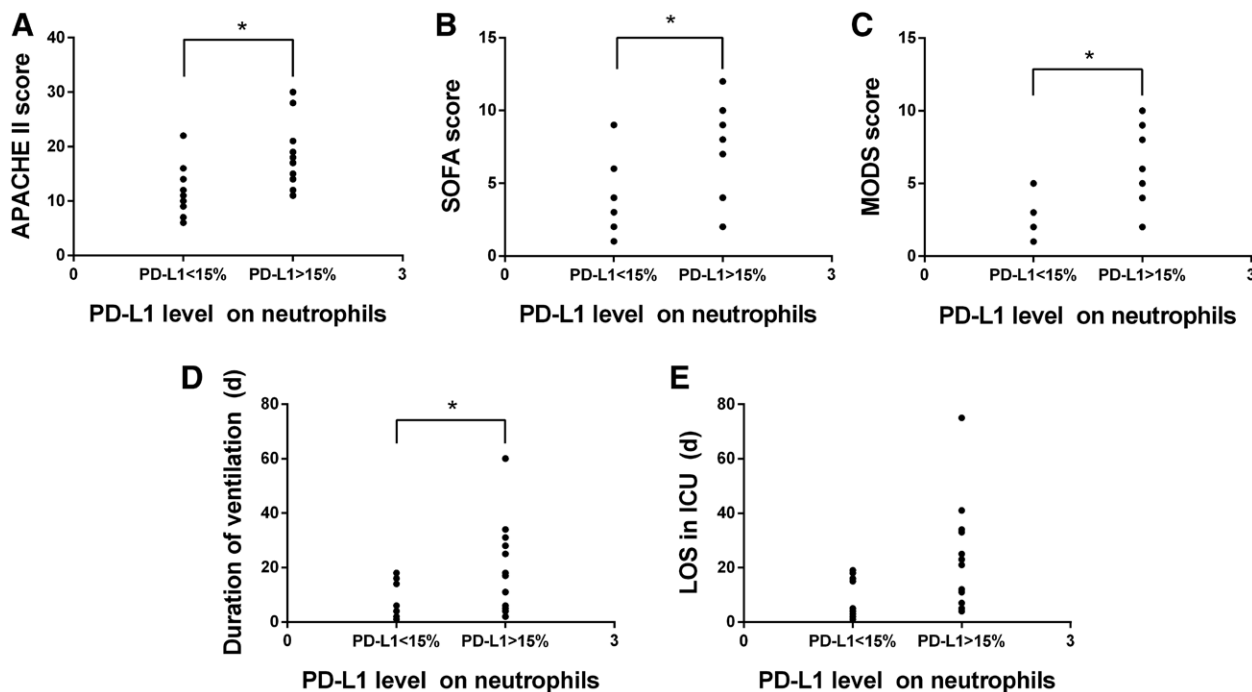


Fig. 9. Disease severity (Acute Physiology and Chronic Health Evaluation [APACHE] II [A], Sequential Organ Failure Assessment [SOFA] [B], and Multiple Organ Dysfunction Syndrome [MODS] [C]), duration of mechanical ventilation (D), and length of stay (LOS) in intensive care unit (ICU) (E) in patients with different programmed death receptor 1 ligand 1 (PD-L1) levels on neutrophils. * $P < 0.05$ by Mann-Whitney U test.

immunosuppression marker for sepsis.³³ Neutrophil PD-L1 may be a better biomarker for immunosuppression for several reasons. First, PD-L1 expression is inducible on neutrophils

during sepsis, whereas HLA-DR is constitutively expressed in monocytes. Second, the PD-L1 level on neutrophils is much higher in patients with severe sepsis than in septic

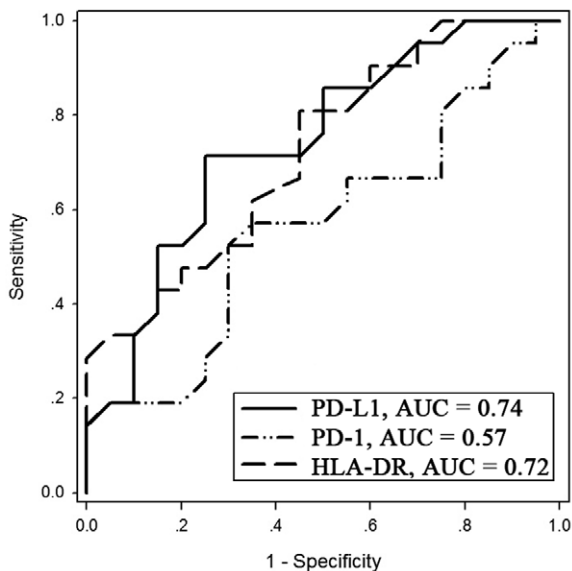


Fig. 10. Receiver operator curve analysis of neutrophil programmed death receptor 1 ligand 1 (PD-L1), monocyte human leukocyte antigen DR (HLA-DR), and lymphocyte programmed death receptor 1 (PD-1) for predicting prognosis of patients with severe sepsis. AUC = area under the curve.

patients without immunosuppression. Third, the predictive value of neutrophil PD-L1 was demonstrated by its correlation with monocyte HLA-DR, disease severity, prolonged mechanical ventilation, and mortality of severe sepsis.

There are several limitations in our study. First, we did not investigate the role of neutrophil PD-L1 in an *in vivo* experiment. Although *in vitro* assays showed that neutrophils might induce lymphocyte apoptosis, there has been no *in vivo* evidence that neutrophil PD-L1 could induce lymphocyte apoptosis in septic animals or patients. Second, lymphocyte abnormalities in sepsis can be manifested in different aspects including apoptosis, a lower proliferation rate, and an inability to secrete interferons. Further studies are required to clarify the inhibitory effect of PD-L1⁺ neutrophils on lymphocyte activity. Third, the sample size was not sufficiently large for biomarker exploration. The diagnostic and prognostic role of neutrophil PD-L1 for sepsis must be further investigated in a well-designed clinical trial. Finally, we did not perform a power analysis because no reference data, such as the SD, were available at the time of this study. Because the PD-L1 level on neutrophils was negligible in cancer patients and healthy volunteers, 10 subjects were sufficient for the comparison according to a *post hoc* analysis.

Conclusions

In conclusion, sepsis can induce PD-L1 up-regulation on neutrophils. PD-L1⁺ neutrophils may be an inhibitory subtype with a specific phenotype and compromised chemotaxis, inducing lymphocyte apoptosis during sepsis. Neutrophil PD-L1 may prove to be a potential biomarker for the diagnosis of sepsis-induced immunosuppression. These

results may provide new insights into the pathogenesis of immunosuppression in sepsis and suggest a biomarker and a therapeutic target for sepsis.

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Competing Interests

The authors declare no competing interests.

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