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High circulating CD39$^+$ regulatory T cells predict poor survival for sepsis patients

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SUMMARY

Background: Sepsis encompasses two phases, the 'hyper'-reactive phase and the 'hypo'-reactive phase. The initial inflammatory stage is quickly counterbalanced by an anti-inflammatory response, which compromises the immune system, leading to immune suppression. Regulatory T cells (Tregs) have been implicated in the pathogenesis of sepsis by inducing immunosuppression; however, the role of CD39$^+$ Tregs in the process of sepsis is uncertain. This study investigated the dynamic levels of CD39$^+$ Tregs and their phenotypic change in sepsis.

Methods: Fourteen patients with systemic inflammatory response syndrome (SIRS), 42 patients with sepsis, and 14 healthy controls were enrolled. Sequential blood samples were used to analyze the numbers of CD39$^+$ Tregs and their phenotypic changes. Survival at 28 days was used to evaluate the capacity of CD39$^+$ Treg levels to predict mortality in sepsis patients.

Results: Sepsis patients displayed a high percentage (3.13%, 1.46%, and 0.35%, respectively) and mean fluorescence intensity (MFI) (59.65, 29.7, and 24.3, respectively) of CD39$^+$ Tregs compared with SIRS patients and healthy subjects. High-level expression of CD39$^+$ Tregs was correlated with the severity of sepsis, which was reflected by the sepsis-related organ failure assessment score ($r = 0.322$ and $r = 0.31$, respectively). In addition, the expression of CD39$^+$ Tregs was associated with survival of sepsis patients ($p < 0.01$). By receiver-operating characteristic (ROC) curve analysis, the percentage and MFI of CD39$^+$ Tregs showed similar sensitivities and specificities to predict mortality (74.2% and 85.1%, and 73.9% and 84.1%, respectively). Using Kaplan–Meier curves to assess the impact of CD39$^+$ Tregs percentage and MFI on overall survival, we found that a high CD39$^+$ Tregs percentage ($p < 0.001$; >4.1%) and MFI ($p < 0.001$; >49.2) were significantly associated with mortality. Phenotypically, CD39$^+$ Tregs from sepsis patients showed high expression of CD38 and PD-1 ($p < 0.01$ and $p < 0.01$ respectively).

Conclusions: Increased expression of CD39$^+$ Tregs was associated with a poor prognosis for sepsis patients, which suggests that CD39$^+$ Treg levels could be used as a biomarker to predict the outcome of sepsis patients.

1. Introduction

Sepsis, defined as a systemic inflammatory response to infection, is associated with high mortality in critically ill patients.[1,2] Patients with sepsis often develop organ dysfunctions, including tissue hypoperfusion and hypoxia, lactic acidosis, oliguria, or altered cerebral function.[3,4] In spite of extensive research efforts over the last 20 years, sepsis remains the leading cause of death in intensive care units (ICUs).[5] As such, research into the pathogenesis of sepsis would lead to a better understanding of this complex syndrome and identify effective therapies.

There is a growing consensus that the initial inflammatory stage is counterbalanced by an anti-inflammatory response after the

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onset of sepsis. This may account for the failure to eliminate pathogens or the development of a secondary infection in sepsis patients.[6] The subsequent immunosuppression might explain why most of the clinical trials using anti-inflammatory strategies have failed to improve the outcome of sepsis.[7] This implies that rectifying immunosuppression may be the key point for the treatment of sepsis.[8] It has long be recognized that $CD4^+CD25^+$ regulatory T cells (Tregs) are important in maintaining self-tolerance and regulating the immune response in both physiological and pathological statuses.[9,10] The population and function of Tregs are considerably diverse, and phenotypically and functionally distinct subsets of Tregs mediate immune suppression through distinct mechanisms.[11] CD39 is a useful marker for $CD4^+CD25^+$ Treg cells; CD39-positive ($CD39^+$) Treg cells have more significant suppression ability compared with CD39-negative ($CD39^-$) Treg cells.[12] Of note, an increase in CD39 expression on Tregs has been observed in patients with HIV infection and is strongly associated with disease progression.[13] Furthermore, an increase in CD39 expression on Tregs has also been identified in tumors and autoimmune diseases.[14]

Although the critical role of $CD39^+$ Tregs in some diseases has been reported, the potential role of $CD39^+$ Tregs in sepsis and its connection with disease progression have not yet been elucidated. Therefore, this study was designed to investigate the frequency of $CD39^+$ Tregs in sepsis and to determine whether the characteristics of this subset are associated with disease severity and mortality.

2. Patients and methods

2.1. Patients and design

Healthy controls and patients with systemic inflammatory response syndrome (SIRS) or sepsis were enrolled consecutively from October 2012 to December 2013. The criteria for SIRS and sepsis were those defined by the American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM).[15] In brief, a diagnosis of SIRS was defined in the presence of two or more of the following criteria: temperature $<36\ ^\circ C$ or $>38\ ^\circ C$, heart rate >90 beats per min in the absence of a pacemaker, respiratory rate >20 times per min or $PaCO_2$ less than 4.3 kPa (32 mmHg), and white blood cell count $>12 \times 10^9$/l or $<4 \times 10^9$/l, or >10% immature band forms. Sepsis was defined as the presence of SIRS associated with infection. Both SIRS and sepsis patients enrolled in this study were patients admitted to the ICU for critical care. SIRS patients were patients without any sign of infection on the first day after elective surgery. Exclusion criteria included age <18 years, pregnancy, malignancy, HIV infection, presence of organ transplantation, and immunosuppressive treatments; SIRS patients who developed sepsis were also excluded during the follow-up period.

Upon admission to the ICU, the following data were recorded for each patient: age, sex, severity of underlying medical conditions, sepsis-related organ failure assessment (SOFA) score, reasons for admission to the ICU, principal diagnosis, vital signs, respiratory parameters, routine blood tests and microbiological culture results. Survival or death was assessed during a follow-up period of up to 28 days. Blood samples were obtained on day 0 from all subjects, on day 5 from sepsis patients, and on the day that the sepsis patients' condition improved. Written informed consent was obtained from the patients or their respective representatives. The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee on Human Research of Beijing 302 Hospital.

2.2. Flow cytometry analysis

Peripheral blood was collected in anticoagulant heparin-containing tubes for flow cytometry staining. In brief, antibodies or isotype control antibodies were added to 100 μl whole blood in the dark for 15 min at room temperature. After red blood cells were lysed, cells were washed twice with phosphate-buffered saline (PBS). The cells were then acquired and analyzed. CD4–fluorescein isothiocyanate (FITC) antibody and CD25–phycoerythrin (PE) antibody were purchased from BD Pharmingen. CD127–Peridinin chlorophyll protein (PerCP) antibody was purchased from BD Bioscience. CD127–Brilliant violet 421 (BV421) antibody was purchased from BioLegend. CD39–Allophycocyanine (APC) and corresponding isotype control antibody were purchased from BD Pharmingen. CD38–FITC and corresponding isotype control antibody were purchased from BD Pharmingen. programmed death 1 (PD-1) –FITC and corresponding isotype control antibody were purchased from eBioscience. The corresponding isotype control antibodies were used for gating the negative cells. All antibodies were used according to the manufacturers' recommended protocols. FACSCalibur and CellQuest software were used to analyze the results.

The lymphocyte population was gated based on morphological parameters on a forward- versus side-scatter (FSC/SSC) plot. Tregs were defined as $CD4^+CD25^+CD127^-$ lymphocytes.[16,17] The mean fluorescence intensity (MFI), which is commonly used to calculate the expression levels of a molecule on cells, was calculated by FACSCalibur and was used to present the expression levels of CD39 molecules on the membrane of Treg cells in this study.

2.3. Statistics

Data analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA); data are expressed as the mean ± standard deviation. Statistical differences between two groups were determined with the Mann–Whitney non-parametric *U*-test. The comparison of data from the same individual was performed using the Wilcoxon matched-pairs *t*-test. The statistical difference in frequency distribution between groups was calculated using Pearson's Chi-square test. Correlation analysis was evaluated by Spearman rank correlation test. Kaplan–Meier curves were plotted to display the impact on survival. Potential differences in overall survival between groups were evaluated with log-rank tests. Receiver-operating characteristic (ROC) curve analysis provided a global and standardized appreciation of the accuracy of a marker for predicting an event in the ICU during follow-up. This statistic allowed a simple comparison of the accuracy of different prognostic scores within the same population. A ROC curve represents the plot of sensitivity against 1 − specificity. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Patient characteristics at baseline

The clinical characteristics of the subjects enrolled are shown in Table 1. Fourteen patients with SIRS, 42 patients with sepsis, and 14 healthy subjects (controls), were enrolled in this study. Both SIRS and sepsis patients were comparable in terms of gender and age with the healthy control subjects ($p = 0.92$ and 0.15, respectively). The SOFA score for sepsis patients was significantly higher than that for SIRS patients ($p < 0.01$). The most common site of infection in sepsis patients was the lung (23 patients, 54.8%), followed by abdomen (16 patients, 38.1%) and genitourinary tract (three patients, 7.1%). Pathogen culture included Gram-negative bacteria (21 patients, 50%), Gram-positive bacteria (nine patients, 21.4%), fungi (three patients, 7.1%), and negative culture (nine patients, 21.4%). The mean ICU stay of sepsis patients was 22.4 ± 8.6 days. The 28-day mortality rate of sepsis patients was 54.7%.

Table 1
Clinical characteristics of the subjects enrolled[a]

	Healthy controls	SIRS	Sepsis	*p*-Value
Number	14	14	42	
Age, years	45.2 ± 10.3	52.4 ± 17.2	49.1 ± 10.2	0.15
Sex, male/female	8/6	7/7	22/20	0.92
SOFA score	-	4.6 ± 2.1	10.9 ± 4.4	<0.01
Site of infection				
Lung	-	-	23 (54.8%)	-
Abdomen	-	-	16 (38.1%)	-
Urinary tract	-	-	3 (7.1%)	-
Pathogen culture				
Gram-negative bacterial	-	-	21 (50%)	-
Gram-positive bacterial	-	-	9 (21.4%)	-
Fungi	-	-	3 (7.1%)	-
Negative culture	-	-	9 (21.4%)	-
WBC, $\times 10^9$/l	-	15.6 ± 3.8	16.2 ± 6.1	0.64
PCT, ng/ml	-	-	5.2 ± 4.6	-
Mechanical ventilation	-	3 (21.4%)	14 (33.3%)	0.61
Renal replacement therapy	-	0 (0%)	10 (23.8%)	0.11
ICU stay, days	-	2.9 ± 0.9	22.4 ± 8.6	<0.01
Mortality, survival/non-survival	-	0% (0/14)	54.7% (19/23)	<0.01

SIRS, systemic inflammatory response syndrome; SOFA, sepsis-related organ failure assessment; WBC, white blood cell count; PCT, procalcitonin.
[a] Data are shown as the number (%) or mean ± standard deviation.

3.2. An increase in circulating CD39$^+$ Tregs is correlated with the severity of sepsis

We first measured the quantity of CD39$^+$ Tregs in the enrolled subjects. The percentage of CD39$^+$ Tregs at admission was significantly higher in sepsis and SIRS patients compared with healthy control subjects (3.13%, 1.46%, and 0.35% respectively; $p < 0.01$). In addition, sepsis patients displayed a higher percentage of CD39$^+$ Tregs than SIRS patients ($p < 0.01$; Figure 1).

Further analysis indicated that the MFI of CD39$^+$ Tregs was lower in SIRS patients compared with that in sepsis patients (29.7 and 59.65, respectively; $p < 0.01$). In the correlation analysis, we found that the percentage and the MFI of CD39$^+$ Tregs were positively correlated with disease severity on day 0, which was reflected by the SOFA score ($r = 0.322$ and $r = 0.31$, respectively). In addition, CD39$^+$ Treg levels on admission significantly stratified sepsis patients into survival and non-survival groups ($p < 0.01$; Figure 2). We also found that the percentage of CD39$^+$ Tregs in infectious ascites ($n = 14$) was higher than that in peripheral blood from patients with sepsis caused by an abdominal infection ($p < 0.05$, Figure 3). In the follow-up study, sepsis patients ($n = 13$) showed significant decreases in CD39$^+$ Treg levels ($p < 0.01$) as they recovered from the disease (Figure 3).

The predictive accuracy value for 28-day mortality was determined by ROC curve analysis. The area under curve (AUC) was 0.85 (95% confidence interval 0.67 to 0.94; $p = 0.001$) for the percentage of CD39$^+$ Tregs and 0.72 (95% confidence interval 0.56 to 0.89; $p = 0.012$) for the MFI of CD39$^+$ Tregs. The percentage of CD39$^+$ Tregs (cut-off point 4.1%) and the MFI of CD39$^+$ Tregs (cut-off point 49.2) showed similar sensitivity and specificity to predict mortality (74.2% and 85.1%, and 73.9% and 84.1%, respectively). Using Kaplan–Meier curves to assess the impact of CD39$^+$ Treg percentage and MFI on overall survival, we found that a high percentage of CD39$^+$ Tregs ($p < 0.001$; log rank 14.05) and CD39$^+$ Tregs MFI ($p < 0.001$; log rank 14.53) were significantly associated with mortality (Figure 4). These data indicate that CD39$^+$ Treg levels are significantly increased in sepsis; the increase is further associated with the severity of sepsis.

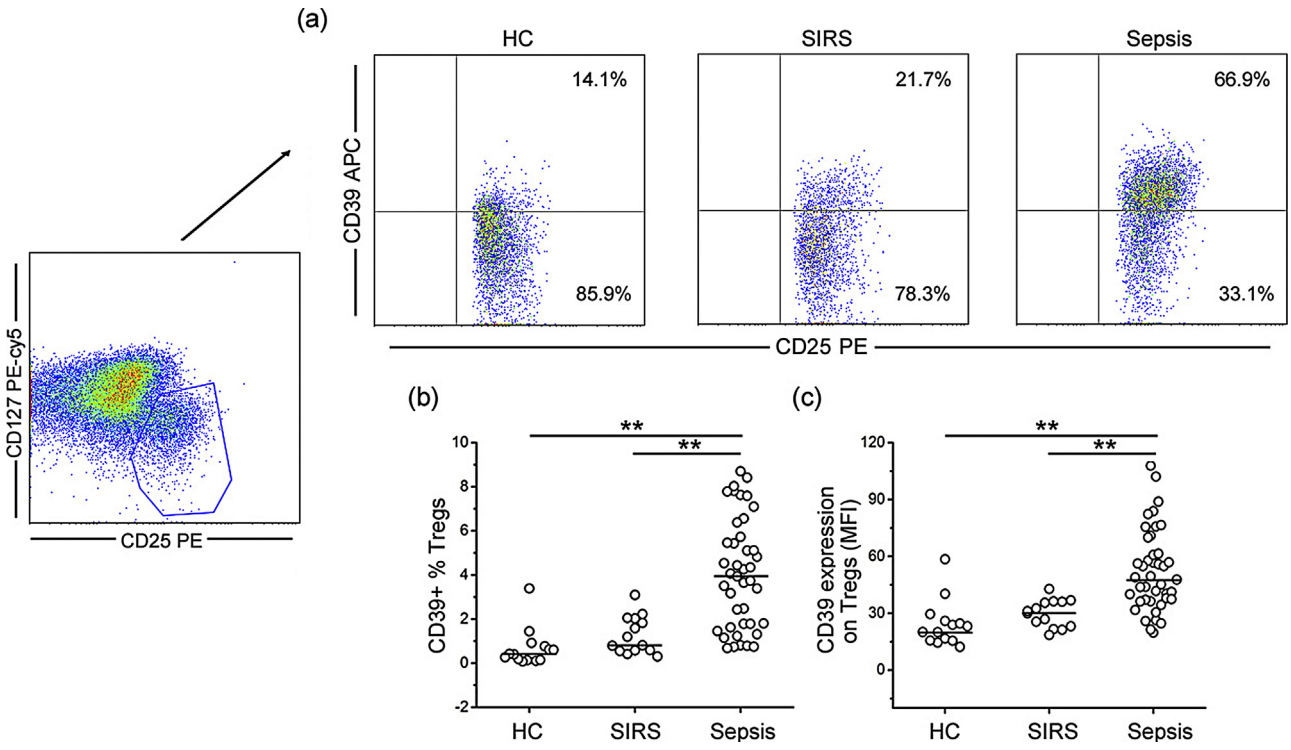

Figure 1. The expression of CD39$^+$Tregs in sepsis patients. (A) The CD39$^+$Tregs defined by CD4$^+$CD25$^+$CD127-CD39$^+$ lymphocytes in healthy control (HC), systemic inflammatory response syndrome (SIRS) and sepsis patients. (B-C) The percentage and the mean fluorescence intensity (MFI) of CD39$^+$Tregs are shown in the enrolled subjects. Each circle represents an individual. ** $P < 0.01$.

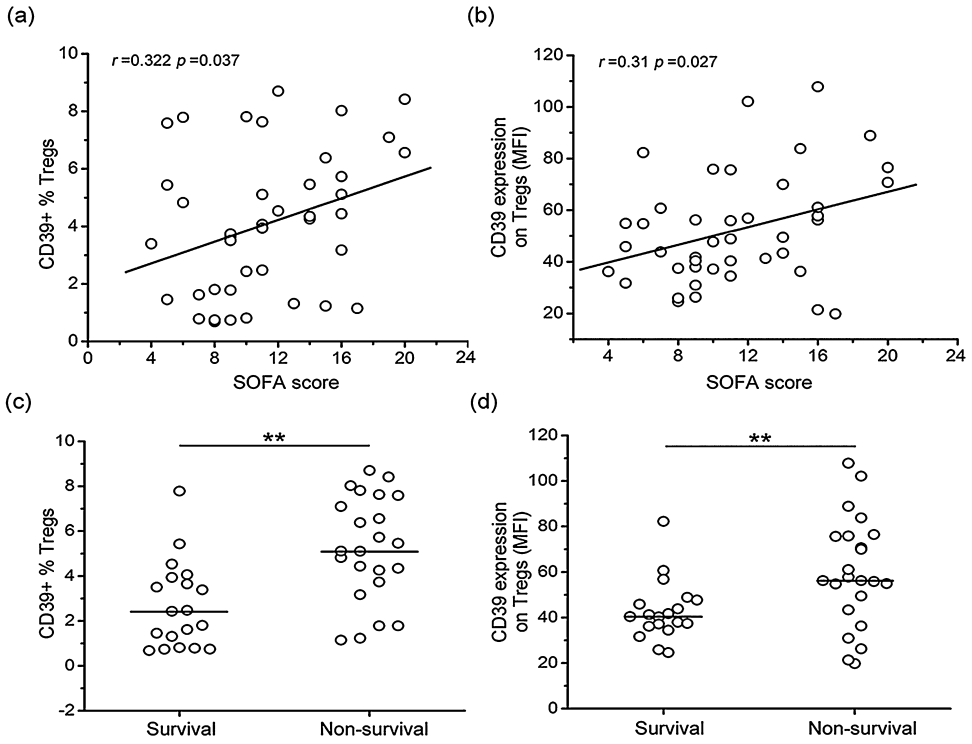

Figure 2. Increased CD39⁺Tregs expression is positively associated with the severity of sepsis. (A-B) The percentage and the mean fluorescence intensity (MFI) of CD39⁺Tregs are correlated with the SOFA score of septic patients. (C-D) The percentage and the mean fluorescence intensity (MFI) of CD39⁺Tregs are associated with mortality of septic patients. Each circle represents an individual.

3.3. Circulating CD39⁺ Tregs exhibit high expression of CD38 and PD-1

CD38 and PD-1 are usually used as phenotypic markers of Treg cells. Thus, we further assessed the co-expression of CD39 and these molecules on Treg cells in patients. In addition to the higher expression of CD38 and PD-1 on CD39⁺ Tregs compared with CD39⁻ Tregs (data not show), the expression of these markers on CD39⁺ Tregs was recorded in healthy, SIRS, and sepsis patients. As shown in Figure 5, cell surface expressions of CD38 and PD-1 were significantly increased in sepsis patients compared with healthy controls ($p < 0.01$ and $p < 0.01$, respectively). Meanwhile, cell surface expressions of CD38 and PD-1 were different between sepsis and SIRS ($p < 0.01$ and $p < 0.01$, respectively). These data indicate that CD39⁺ Tregs in sepsis patients show a high level of activation and are prone to exhaustion.

4. Discussion

Sepsis is considered a race to the death between the pathogens and the host immune system; maintaining a proper balance between pro- and anti-inflammatory pathways determines the fate of the sepsis patient. In addition to the overwhelming pro-inflammatory immune response responsible for sepsis, sepsis patients also appear to manifest rapid immune dysfunctions consistent with a state of immune suppression. This state is typically characterized by defects in both innate and adaptive immune responses, and leads to secondary infection and increased mortality.[18,19] Tregs suppress both innate and adaptive immunity, and are regarded as a marker of the prognosis or recurrence of the underlying disease.[20,21] Thus, measurement of the Tregs percentage might represent a simple and valuable surrogate marker of

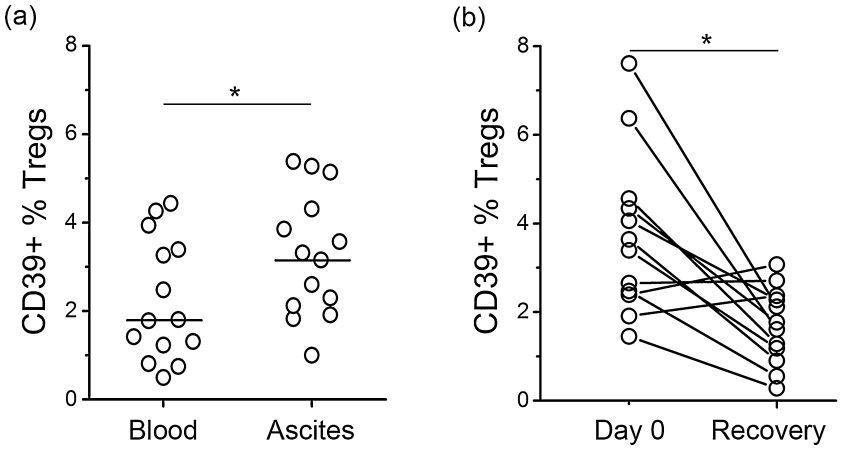

Figure 3. Dynamic expression of CD39⁺Tregs in sepsis patients. (A) Differential expression of CD39⁺Tregs in blood and ascites from patients with sepsis caused by abdominal infection. (B) The decreased CD39⁺Tregs level in blood correlates with improvement of septic patients. Each circle represents an individual. * $P < 0.05$; ** $P < 0.01$.

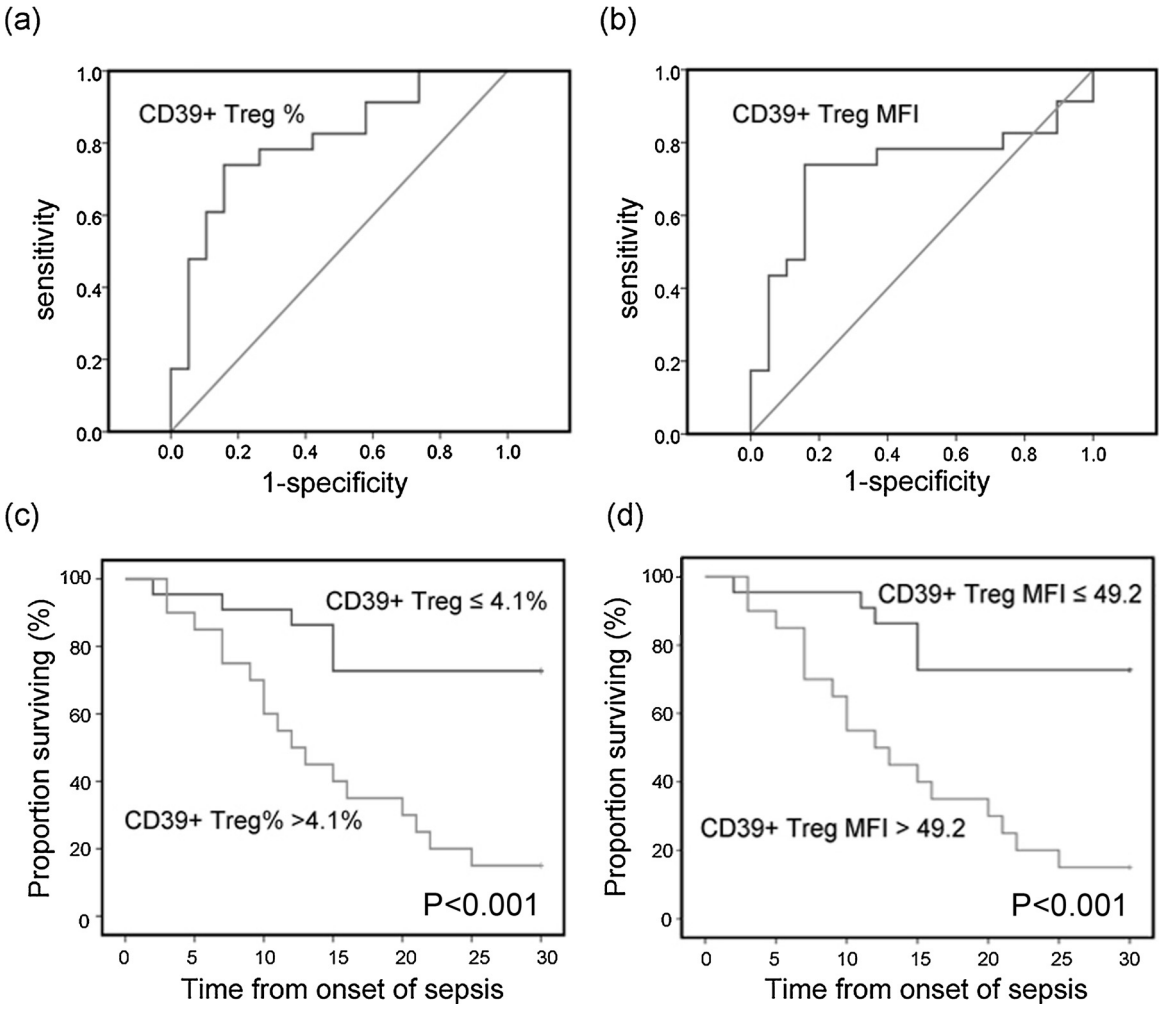

Figure 4. The CD39$^+$Tregs level is a prognostic marker for sepsis patients. (A-B) Receiving operating characteristic (ROC) curve of CD39$^+$Tregs as percentage of positive cells or mean fluorescence intensity (MFI) at day 0 for predicting mortality. Area under curve: 0.805 for CD39$^+$Tregs percentage, and 0.728 for CD39$^+$Tregs MFI. (C-D) Kaplan-Meier survival curves of septic patients showing that septic patients with high CD39$^+$Tregs level (>4.1%) and MFI (>49.2) have an increased mortality as compared to patients with low CD39$^+$Tregs level and MFI ($P < 0.001$ and $P < 0.001$ respectively). Follow-up period: 28 days.

lymphocyte anergy. While several potential abnormalities have been identified, it appears that Tregs represent a double-edged sword in infection; the role of Tregs in bacterial sepsis remains controversial because of conflicting results in antibody-mediated depletion experiments.[22,23]

For sepsis patients, timely testing of the direction of the inflammatory balance may be helpful to correct their immune response. Meisel et al. reported that monocytic human leukocyte antigen-DR (mHLA-DR)-guided granulocyte-macrophage colony-stimulating factor (GM-CSF) therapy was helpful for restoring

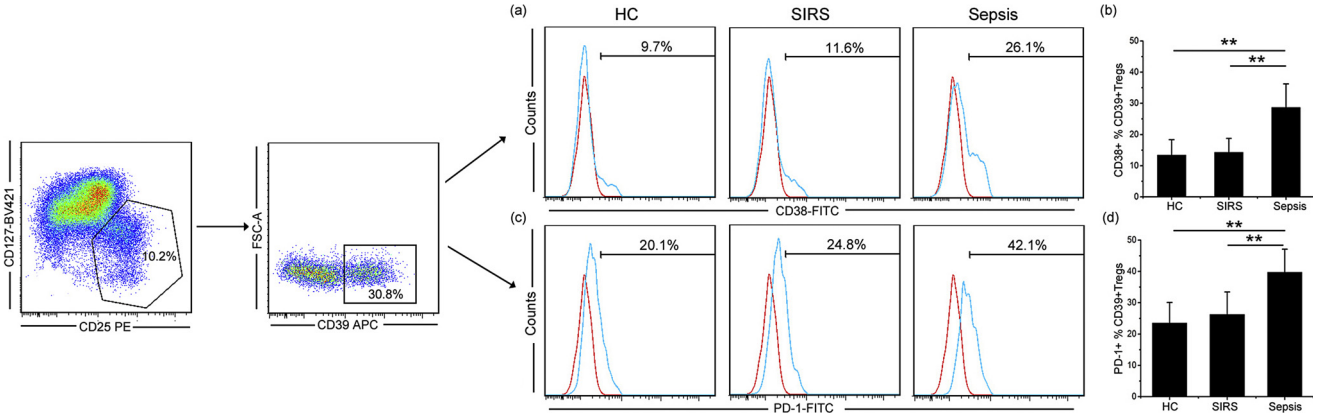

Figure 5. CD39$^+$Tregs from septic patients express high levels of CD38 and PD-1. (A) Representative dot plots showing the co-expression of CD39 and CD38, PD-1 on Tregs. (B) Pooled data indicating that CD39$^+$Tregs in septic patients express high levels of CD38 and PD-1. * $P < 0.05$; ** $P < 0.01$.

monocytic immunocompetence, reducing the duration of mechanical ventilation and ICU stay for sepsis patients;[24] this indicated that biomarker-guided immunotherapy administered to sepsis patients in the correct immune phase is a potential major advance in the treatment of sepsis. Unfortunately, biomarkers that can be used to identify those patients who are at the highest risk of mortality and who thus might best benefit from such therapies are limited.

Recent studies showed that $CD39^+$ Tregs constitute a special subset of activated Tregs, possessing more powerful immune suppression ability.[25] However, the dynamics of $CD39^+$ Tregs expression and their association with the progression of sepsis remain unknown. Our data provide evidence to support the association between $CD39^+$ Tregs and the progression of sepsis. First, circulating levels of $CD39^+$ Tregs increased significantly in sepsis patients compared with SIRS patients and healthy control subjects. Second, there was a significant positive correlation between $CD39^+$ Treg expression and the SOFA score. Furthermore, ROC analyses indicated that the percentage or the MFI of $CD39^+$ Tregs could serve as a biomarker for predicting the outcome of sepsis (AUC 0.85 and 0.72, respectively). In contrast to our results, recent work by Kühlhorn et al. showed that the suppressive capacity of $FoxP3^+$ Tregs was not sufficient to control overwhelming inflammation and early mortality, but was a prerequisite for the recovery from severe sepsis.[26] This suggests distinct phenotypic and functional subsets of Tregs mediate immune suppression through distinct mechanisms.

It is established that $CD39^+$ Tregs play a central role in inducing immunosuppression;[27] however, until now, very few studies have investigated the phenotypic changes in $CD39^+$ Tregs in patients with sepsis. It has been demonstrated that CD39+ Tregs express high levels of functional markers, including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), HLA-DR, and Ki-67, compared with $CD39^-$ Tregs during hepatitis B virus infection.[28] Furthermore, in autoimmune hepatitis, dysfunctional $CD39^+$ Tregs were found to correlate with aberrant control of Th17 cells.[29] However, Tregs are heterogeneous, and there is a discrepancy between their physiology and the pathological condition. In this study, the phenotype change of $CD39^+$ Tregs was investigated in healthy controls, SIRS, and sepsis patients. Our data show that sepsis patients exhibit higher expression of functional markers such as CD38 and PD-1, which may reflect that, in addition to the change in the $CD39^+$ Tregs population, the function of $CD39^+$ Tregs is also remodified during sepsis. The increased expression of CD38 and PD-1 suggests that $CD39^+$ Tregs are prone to activation and exhaustion.

This study is not without limitations. By ROC analyses, this study indicates that $CD39^+$ Tregs could serve as a biomarker for predicting the mortality of sepsis patients; however more clinical studies and additional animal studies are needed to elucidate the associated mechanism. Further, although significantly different levels of $CD39^+$ Tregs were found between blood and infectious ascites, we did not compare the other sites of infection with blood because of the limited samples, thus these data should be referred to abdominal sepsis. Finally, we compared these ICU patients based only on whether they were SIRS or sepsis patients because of the relatively small sample size. If we increased the number of subjects and stratified the data for different severities of disease such as SIRS, sepsis, severe sepsis, and sepsis shock, the relationship of $CD39^+$ Treg expression with disease severity might be more important.

In conclusion, our data show that sepsis is associated with a profound dysfunction of $CD39^+$ Tregs. The percentage and MFI of $CD39^+$ Tregs could serve as a biomarker for predicting the outcome of sepsis. These data indicate that $CD39^+$ Tregs may be a potential target for rectifying the dysfunctional inflammatory response in sepsis.

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Conflict of interest: The authors declare that they have no competing interests.

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