JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Biomarkers

Research Article

The Level of Regulatory T Lymphocytes may be Correlated with Outcomes in Early Sepsis

Tsai-Hsia Hong[1], Chin-Hao Chang[2], Jo-Wei Shen[3], Wen-Je Ko1[3], Chun-Ta Huang[3], Sun-Liang Yu[4], Yih-Sharng Chen[*1]

[1]*Department of Surgery, National Taiwan University Hospital, Taiwan*

[2]*Department of Medical Research, National Taiwan University Hospital, Taiwan*

[3]*Department of Traumatology, National Taiwan University Hospital, Taiwan*

[4]*Department of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taiwan*

**Corresponding author: Dr. Yih-Sharng Chen, Department of Surgery, National Taiwan University Hospital, Taipei 100, Taiwan, Tel: 886-2-23123456 ext.65066; Fax: 886-2-2395-6934; E-mail: yschen1234@gmail.com*

Received: 11-26-2014

Accepted: 11-27-2014

Published: 12-19-2014

Abstract

Severe sepsis is associated with high mortality; however, the mortality of the secondary infection after severe sepsis is often higher than that of primary infection. A weaker pro-inflammatory response was found in survivors in primary inflection. The levels of pro-inflammatory cytokines in mid-term survivors, late death due to the secondary infection, were significantly higher than those in long-term survivors. Our hypothesis is that regulatory T cells (Tregs) suppress adaptive immune response in non-survivors and mid-term survivors. Therefore, the levels of Tregs between non-survivors and survivors, as well as between mid-term and long-term survivors in severe sepsis patients were examined to verify the hypothesis. Total 24 cases of severe sepsis, with outcomes of the early death (n = 5), mid-term survivors (n = 6), and long-term survivors (n = 13) were enrolled. The levels of lymphocytes, Tregs, monocytes, granulocytes, and neutrophil CD64 molecules were analyzed on days 0 (within 12 hours after the onset of the first organ failure due to sepsis), 1, 2, and 3 by flow cytometry and the levels of each variable were compared between groups by the mixed model. The recovery of lymphocytes was only observed The level of Tregs was significantly different between the early death and survivors, the early death and long-term survivors, in long-term survivors, as well as mid-term and long-term survival groups. The levels of neutrophil CD64 molecules indicated the ongoing infection. Results indicate that Tregs might affect immune homeostasis in patients with severe sepsis, influencing outcomes of early death, mid-term survival, and long-term survival.

Keywords: Severe Sepsis; Regulatory T Lymphocytes; Lymphocytes; Lymphocytopenia; Monocytes.

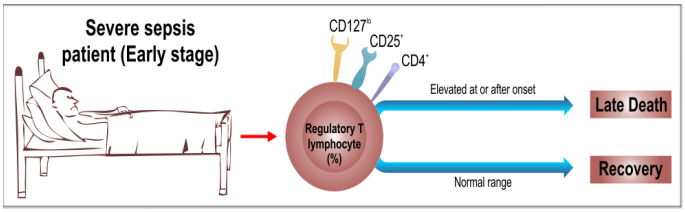
Abbreviations

CARS : Compensatory Anti-Inflammatory Response Syndrome;
LTSG : Long-Term Survival Group;
MTSG : Mid-Term Survival Group;
SIRS : Systemic Inflammatory Response Syndrome;
Treg : Regulatory T Lymphocyte

Introduction

In intensive care units (ICUs), sepsis is one of the main causes of death and is often diagnosed on the basis of clinical symptoms, laboratory examinations, and lymphocytopenia [1]. The mechanism of systemic infection-induced lymphocytopenia is not clear. Microorganism infection induces not only an inflammatory response but also a release of anti-inflammatory factors, resulting in a heightened inflammatory reaction leading to early death, a balance between inflammatory and anti-inflammatory factors resulting in recovery [2]. Neutrophil CD64 molecules have been used as the indication of bacterial infection [3]. Our previous results indicate that a weaker pro-inflammatory response was found in survivors compared to non-survivors in the primary infection; furthermore, those died due to the secondary infection (mid-term survivors) did not have a more pronounced IL-10 response compared to long-term survivors in the early phase. However, the levels of pro-inflammatory cytokines between mid-term and long-term survivors were significantly different [4]. Immunoparalysis is considered as the main cause of the secondary infection in animal models [5]. No clinical evidence has been presented; furthermore, the duration of immune suppression is not known. We hypothesize that regulatory T cells (Tregs) suppress adaptive immune response in non-survivors, particularly for the late death from the secondary infection, see Figure 1. We measured the levels of lymphocytes, Tregs, monocytes, and granulocytes in severe sepsis patients to verify the correlation of Tregs to outcomes of early death, late death, and recovery. The ability to predict late death in the early phases of sepsis would help physicians develop new management strategies for these patients.

Figure 1. The hypothesis of regulatory T lymphocyte levels related to the outcomes of severe sepsis patients.



Methods

Study population

This study included sepsis patients admitted to our institution between December 10, 2010 and December 10, 2011 who had highly probable or proven infection and at least 3 of the following systemic inflammatory response syndrome (SIRS) criteria: body temperature, >38°C or <36°C; heart rate, >90 beats/min; breathing frequency, ≥20 breaths/min, $PaCO_2$, <32 mmHg, or ventilator use; leucocyte count, >12000/mm^3 or <4000/mm^3, or band forms, >10%; acute altered mental status; hyperglycemia without a history of diabetes, blood glucose > 120 mg/dL. Other inclusion criteria were age ≥18 years, ICU admission, and at least one sepsis- or septic shock-induced organ failure. Septic shock was defined as sepsis with hypotension refractory to fluid challenge. Pregnant women, patients who refused resuscitation, those with known or suspected human immunodeficiency virus infection, and those with known or suspected underlying immune deficiency were excluded. The zero time point (day 0) was designated within 12 hours after the first sepsis-induced organ failure. Blood was collected on days 0, 1, 2, and 3. This study was approved by the National Taiwan University Hospital Research (NTUH) Ethics Committee.

Treatment for sepsis was managed by the drainage of the possible abscess, proper antibiotic agents for underlying etiologies of microorganisms, and hemodynamic management for the perfusion. The hemodynamic management included adequate volume with inotropic support with/without norepinephrine.

Flow Cytometric Analysis.

Blood was collected in an ethylenediaminetetraacetic acid vacutainer tube and was separately stained with CD4FITC/CD127PE/CD25APC and Quantibrite CD64PE/CD45PerCP according to the manufacturer instructions (Becton Dickinson, San Jose, California, USA). For every test, 20,000 live leukocytes were collected and analyzed using a BD FACSCalibur flow cytometer with Cell Quest software version 3.2 (Becton Dickinson, San Jose, California, USA). The lymphocytes were further plotted on CD4 fluorescence intensity versus size scatter and gated on CD4+ lymphocytes for CD127 PE and CD25 dot plot analysis. The percentage of CD4+CD25+CD127-/weak+ cells of CD4+ lymphocytes presented the percentage of regulatory T lymphocytes in peripheral blood.

Statistical Analysis

Patient outcomes were classified as death due to severe sepsis (early death) or survival. Those who survived were further divided into the mid-term survival group (MTSG, survival through severe sepsis but death or continued hospitalization within 6 months) and the long-term survival group (LTSG, recovery). One patient in the MTSG died on day 176, and another one was transferred to the ICU of another hospital after a 6-month hospitalization period. Six-month was the longest traceable period for enrolled cases; therefore, it was used as the cut-off value to categorize patients into the MTSG and LTSG. Patients in the LTSG were discharged from the hospital in good health and survived for >6 months. The levels of lymphocytes, monocytes, granulocytes, Tregs and neutrophil CD64 molecules were compared between the early death and survivors, the death and LTSG, as well as MTSG and LTSG using the mixed model. Statistical analyses were performed using SAS, version 9.2 (SAS Institute Inc., Cary, North Carolina, USA).

Results

Patient Characteristics

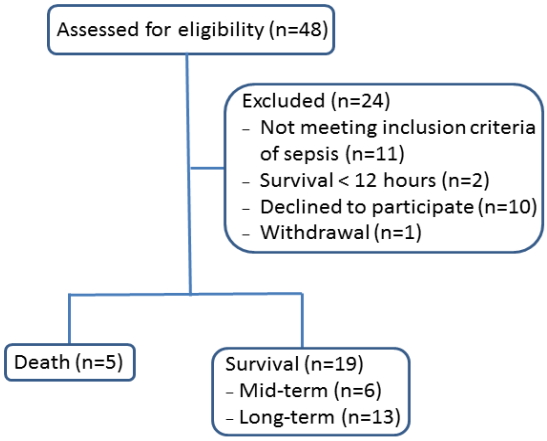
Characteristics of the 24 enrolled patients are listed in Table 1. The flow chart of screening 48 cases is presented in Figure 2 Five patients died due to sepsis on days 2, 5, 7, 10, and 10, and blood cultures were positive in all cases (100%, the early death group). Five patients died due to secondary infection on days 19, 44, 45, 49, and 176, and blood cultures before death were negative (the MTSG). One patient with continued hospitalization for 6 months was diagnosed as having necrotizing pancreatitis with persistent multiple intra-abdominal abscesses. This patient was transferred to the ICU of another hospital after a 6-month stay at our institution. The mean ± standard deviation age was lower in the MTSG (64.8 ± 13.5 years) than in the LTSG (78.0 ± 11.5 years). The positive blood culture rates in the MTSG and LTSG were 83.3% and 38.5%, respectively. The infection was confirmed by the analysis of neutrophil CD64+ quantification.

Table 1. Characteristics of severe sepsis patients.

Diagnosis	Death (N=5)	Survivors	
		Mid-Term (N=6)	Long-Term (N=13)
Female/male	1/4	1/5	9/4
Age (years)	72.0±13.6	64.8±13.5	78.0±11.5
Pneumonia	2	3	5
K. pneumonia	0	0	0
MSSA	0	0	1
MRSA	1	1	0
E. cloacae, K. pneumonia	1	0	0
S. marcescens	0	0	1
E. coli	0	1	0
Urinary tract infection	1	0	3
K. pneumonia	1	0	0
E. coli	0	0	1
E. coli, A. baumannii	0	0	1
Bed sore infection	1	1	0
M. morganii	1	0	0
C. albicans	0	1	0
Cellulitis	0	0	1
Intra-abdominal abscess	1	2	4
E. coli	0	1	0
A. baumannii, S. maltophilia	0	1	0
A. lwoffii	0	0	1
K. pneumonia	1	0	0
Positive blood culture rate	100%	83.3%	38.5%

Death: cases who died due to sepsis; Mid-term survival: cases who survived sepsis but died or were in continued hospitalization within six months; Long-term survival: cases that had complete recovery and survived > 6 months.

Figure 2. Flow chart of the cases included in this study.



Changes in lymphocyte and granulocyte levels in the three outcome groups

Comparisons of lymphocyte, monocyte, and granulocyte levels between the early death and survival groups, the early death and LTSG, as well as the MTSG and LTSG are presented in Figure 3. The level of Tregs is presented as the percentage of CD4+ lymphocytes and neutrophil CD64 quantification is presented as the number of CD64 molecules per neutrophil. The survival group showed recovery of lymphocyte and granulocyte levels. However, the patterns of lymphocyte and granulocyte recovery differed between the MTSG and LTSG. Comparisons of lymphocytes, monocytes, granulocytes, Tregs, and neutrophil CD64 molecules levels between the early death and survival, the early death and LTSG, as well as MTSG and LTSG by the mixed model are listed in Table 2. The lymphocyte level differed significantly between the early death group and the LTSG ($p = 0.03$). The monocyte level did not differ significantly between the early death group and the LTSG. The granulocyte level was significantly different between the early death group and the LTSG ($p = 0.05$).

Table 2. Comparisons of lymphocyte, monocyte, granulocyte, regulatory T lymphocytes (Tregs) and neutrophil CD64 molecule levels between the early death and survival, the early death and long-term survival groups, as well as mid-term and long-term survival groups using a mixed model.

Variable	Death vs. Survival	Death vs. LTSG	LTSG vs. MTSG
Lymphocytes	$p = 0.16$	$p = 0.03$	$p = 0.16$
Monocytes	$p = 0.13$	$p = 0.10$	$p = 0.13$
Granulocytes	$p = 0.13$	$p = 0.05$	$p = 0.13$
Tregs	$p = 0.02$	$p = 0.05$	$p = 0.02$
Neutrophil CD64	$p = 0.80$	$p = 0.47$	$p = 0.80$

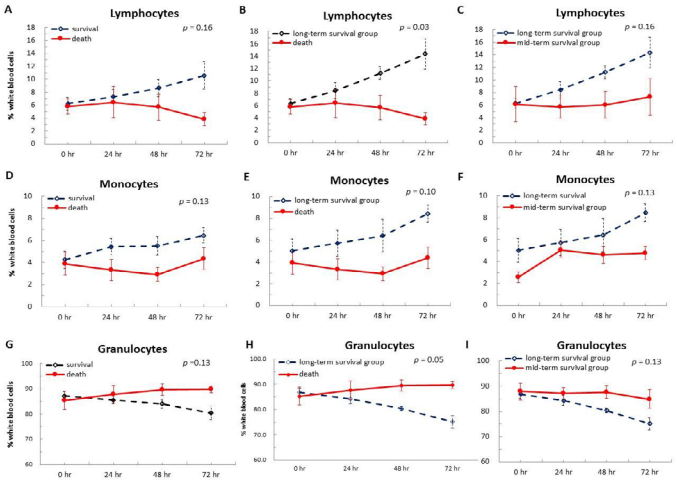


Figure 3. Percentage comparisons of lymphocytes (A, B, C), monocytes (D, E, F), and granulocytes (G, H, I) between the early death and survival groups (A, D, G), the early death and long-term survival (B, E, H), as well as the mid-term and long-term survival groups (C, F, I). The data plotted are the mean ± SEM of each group. The p-value of each comparison is stated.

Changes in Treg levels in the three outcome groups

Comparisons of the Treg and neutrophil CD64 molecule levels are presented in Figure 4 and results of statistical analysis are presented in Table 2. The Treg level was the only significant factor between the early death and survival (p = 0.02), the early death group and the LTSG (p = 0.05), as well as the MTSG and LTSG (p = 0.02). No significant difference in neutrophil CD64 molecule level was found in group comparisons.

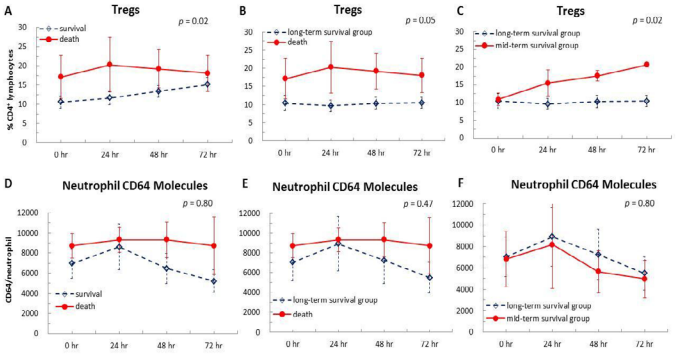


Figure 4. Comparisons of regulatory T lymphocyte, Treg, (A, B, C) and neutrophil CD64 molecule (D, E, F) levels between the early death and survival groups (A, D), the early death and long-term survival (B, E), as well as the mid-term and long-term survival groups (C, F,). The data plotted are the mean ± SEM of each group. The p-value of each comparison is stated.

Discussion

The lymphocyte, Treg, and granulocyte levels differed significantly between patients in the early death group and the LTSG. The Treg level was also significantly different between patients in the early death and survival, and the MTSG and LTSG. Venet et al reported that the lymphocyte level was significantly lower in patients with septic shock than in normal individuals, and these low levels were maintained for 48 hours [6]. However, patients were not classified according to outcome in that study. In our study, the results for the lymphocyte level in all patients are similar to those of the study by Venet et al. However, on classification of patients into three groups, the distinction in lymphocyte levels became clear. The LTSG group patients showed recovery from lymphocytopenia, whereas patients who died immediately from sepsis and those who died later due to secondary infection did not show lymphocyte recovery. Accordingly, early recovery from lymphocytopenia might be essential for survival.

Patients generally experience SIRS in response to a severe pro-inflammatory stimulus. This induces a compensatory anti-inflammatory response syndrome (CARS), which contains the hyper-inflammatory response. Persistent CARS is referred to as immunoparalysis. $CD4^{+}CD2^{5+}CD127^{-/low}$ Tregs account for 8–10% of CD4+ T lymphocytes and are very effective in suppressing the immune response [7]. The Treg level has been considered a potential marker of immunoparalysis; however, there is no clinical evidence in support of this hypothesis [8,9] In an immunohistochemical comparison of spleens obtained from patients who died due to active severe sepsis and control spleens from patients who died due to brain death or trauma, extensive depletion of splenic $CD4^{+}$, $CD8^{+}$, and human leucocyte antigen class II cells was observed in the sepsis group. The Treg level was approximately 2-fold higher in patients who died due to sepsis compared to control patients [10]. The lymphocytopenia and increased Treg level seen in patients who died due to sepsis correspond to the results of our study.

In our study, the Treg level was an important distinguishing factor between the MTSG and LTSG during the first few days after severe sepsis. Venet et al also studied this level in 30 septic shock patients and 17 normal controls on days 3 and 7 after septic shock [8]. The Treg percentage was significantly higher in patients with sepsis than in control patients. They hypothesized that Tregs reduce lymphocyte proliferation in patients with sepsis but did not perform a clinical evaluation [11]. Hein et al examined Treg profiles between patients with septic shock and normal controls on days 1, 3, 5, and 7 after admission [12]. Their results were in contrast to the concept that the Treg percentage is inversely associated with the severity of shock. They explained that this conflict was attributable to the small number of cases in the death group. Hein et al did not classify patients who survived into an MTSG and LTSG. Similarly, we also found that the Treg percentage started to increase from day 1 in patients who survived; however, patients who survived were further divided into two groups in our study, allowing for discrimination between the Treg percentages in

these two groups.

In conclusion, we present clinical evidence that the Treg levels were significantly different in outcomes of early death, recovery, and late death due to secondary infection. This will help in the determination of management strategies for each patient group. Nonetheless, the limitation of this study is the small sample size.

Conflict of Interest Statement

There is no conflict of interest to declare.

Acknowledgments

This work was supported in part by NTUH grant no. 100-001494 and 103-S2380. The authors acknowledge the statistical assistance provided by the National Translational Medicine and Clinical Trial Resource Center (supported by the National Science Council of Taiwan; NSC101-2325-B-002-078).

References

1. Derek C, Angus DC. The search for effective therapy for sepsis back to the drawing board?. JAMA. 2011, 306(23): 2614-2615.

2. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med. 2003, 348(20): 138-150.

3. Icardi M, Erickson Y, Kilborn S, Stewart B, Grief B et al. CD64 index provides simple and predictive testing for detection and monitoring of sepsis and bacterial infection in hospital patients. J Clin Microbiol. 2009, 47(12): 3914–3919.

4. Hong TH, Chang CH, Ko WJ, Lin CF, Liu HH et al. Biomarkers of early sepsis may be correlated with outcome. J Transl Med. 2014, 12: 146.

5. Sakaguchi S, Ono M, Setoguchi R, Yagi H, Hori S et al. Fox-3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol Rev. 2006, 212: 08-27.

6. Venet F, Davin F, Guignant C, Larue A, Cazalis MA et al. Early assessment of leukocyte alterations at diagnosis of septic shock. Shock. 2010, 34(4): 358-363.

7. Shevach EM. Regulatory T cells in autoimmunity. Annu Rev Immunol. 2000, 18: 423-449.

8. Shubin NJ, Monaghan SF, Ayala A. Anti-inflammatory mechanisms of sepsis. Contrib Microbiol. 2011, 17: 108-124.

9. Hall TC, Bilku DK, Al-Leswas D, Horst C, Dennison AR. Biomarkers for the differentiation of sepsis and SIRS: the need for the standardisation of diagnostic studies. Ir J Med Sci. 2011, 180(4): 793-798.

10. Boomer JS, To K, Chang KC, Takasu O, Osborne DF et al. Immunosuppression in patients who die of sepsis and multiple organ failure. JAMA. 2011, 306(23): 2594-2605.

11. Venet F, Chung C-S, Kherouf H, Geeraert A, Malcus C et al. Increased circulating regulatory T cells (CD4+CD25+CD127-) contribute to lymphocyte anergy in septic shock patients. Intensive Care Med. 2009, 35(4): 678-686.

12. Hein F, Massin F, Cravoisy-Popovic A, Barraud D, Levy B et al. The relationship between $CD4^{+}CD25^{+}CD127^{-}$ regulatory T cells and inflammatory response and outcome during shock states. Crit Care. 2010, 14(1): R19.