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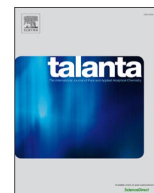

Combined CD25, CD64, and CD69 biomarker panel for flow cytometry diagnosis of sepsis

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ABSTRACT

Sepsis is a highly prevalent syndrome in the United States. The use of cell surface markers, as an effective tool to diagnosis sepsis, has been widely investigated. However, the study of the combination of multiple biomarkers to achieve higher diagnosis accuracy is rare. This study, the panel combined with CD25, CD64, and CD69 was constructed and better diagnosis ability was observed. Septic patients (n = 40), with the mean age of 61 ± 14, were enrolled in this study, along with healthy volunteers (n = 10), included as a control group. All blood samples were measured by flow cytometry based on different subtypes of leukocytes, including neutrophils, monocytes, and lymphocytes. Antigen expression and the antigen positive cell population were reported separately based on cell types. CD64 was the best biomarker in predicting sepsis. The area under Receiver Operating Characteristic (ROC) curve (AUC) was 0.928 and 0.934 for neutrophil CD64 expression and CD64 + neutrophil population, respectively, indicating an excellent diagnosis ability for sepsis. A significant increase was also observed in the populations of CD25 + lymphocytes and CD69 + lymphocytes (p = 0.02 and 0.042, respectively; 95% confidence interval). A panel of combined CD25, CD64, and CD69 was constructed. The parameters of neutrophil CD64 expression, CD64 + neutrophil population, CD25 + lymphocyte population, and CD69 + lymphocyte population were included. The AUC of the ROC curve for this new constructed panel was 0.978. This result indicated that the combination of CD25, CD64, and CD69 outperformed each one of the single parameters in predicting sepsis alone.

1. Introduction

Sepsis is an extreme response to infections, and is a leading cause of death in the United States [1]. The number of cases of sepsis worldwide is still unknown since the data of developing countries is difficult to collect [2]. However, in the United States, Intensive Care Units (ICUs) receive more than 5 million septic patients every year [3,4], with an overall cost of $24 billion [4]. The unplanned readmission rate following sepsis hospitalization is larger than other diseases including Acute Myocardial Infarction (AMI), heart failure, pneumonia, *etc* [5]. Even for patients who recover from sepsis, there are significant health care and personal implications due to long-term physical, psychological, and cognitive disabilities [6]. In the case of false negative sepsis diagnosis, there is an increase in patient mortality with delayed treatment. The most widely used treatments for sepsis currently are intravenous fluids and antibiotics [7]. However, false positive diagnosis of sepsis, especially in the early stage, causes the overuse of antimicrobial agents. This overuse leads to increased microbial drug resistance. Therefore, rapid and accurate identification of sepsis from suspected infections is needed.

Currently, quick diagnosis of sepsis from suspected infections can be confirmed by quick Sepsis Related Organ Failure Assessment (qSOFA) scores [8]. qSOFA incorporates several vital signs of patients, including respiratory rate of 22/min or faster, altered mentation, and systolic blood pressure of 100 mm Hg or less [8]. A qSOFA score ≥ 2 indicates sepsis, but sepsis can be present without qSOFA score ≥ 2 because different forms of organ dysfunction may be present than those assessed using the qSOFA, such as hyperbilirubinemia, hypoxemia, coagulopathy, or renal failure [9]. Therefore, during hospitalization, blood culture is typically required for the validation of sepsis, which takes at least 24–48 h [10,11]. However, in the case of early sepsis, the yield of positive blood culture is very low. On average, only 40% of septic patients would show positive results [12]. Biomarkers have been investigated as means of aiding early diagnosis and, therefore, early

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initiation of appropriate therapy in patients with sepsis and sepsis-related diseases [13]. Over two hundred biomarkers have been proposed, but only a few are potentially useful in clinical practice [13]. C-reactive protein (CRP) is synthesized principally by hepatocytes in response to stimulation by cytokines [12]. Hence its level is increased in most forms of acute and chronic inflammatory states including sepsis syndromes, making it a widely used biomarker of infection. However, a lack of specificity is the main drawback of CRP, since its level may be increased in many inflammatory and infectious disorders [12]. Also, only changes in CRP level, rather than the absolute CRP values, are useful in predicting sepsis. This measurement strategy prolongs analysis time, which is significant in the efficacy of sepsis treatment since every hour of delay in appropriate antibiotic therapy increases mortality by 5–10% [14].

Another biomarker that has been proposed is Procalcitonin (PCT). Although PCT usually has higher sensitivity compared with CRP, the specificity is still low [14]. In addition, false-positive and false-negative results are common [15].

The hyperstimulation of cells caused by sepsis may guide us in the correct way on diagnosis and treatment design [16,17]. Cell surface markers, such as CD64, CD25, and CD69, are promising markers for sepsis detection, diagnosis, and prognosis. Hirsh *et al.* showed that the number, as well as the expression, of both CD64 + neutrophils and monocytes increased significantly in septic patients [18]. However, the single parameter described in their work was not strong enough to be used in the field of prognosis of sepsis clinically. CD4 +CD25 + T-lymphocytes, an important subset of lymphocytes for the control of immune response, have been studied widely. Although the role of these cells in sepsis is still not completely understood [19], the upregulation of the cell population has been observed. Rodrigo *et al.* [20], Francois *et al.* [21], Feng-Ying Leng *et al.* [22], and Fabienne *et al.* [19] reported that the percentage of CD4 +CD25 + T-lymphocytes of septic patients significantly increased. Furthermore, Azza *et al.* [23] and Steven *et al.* [24] found a statistical difference between septic patients and healthy controls of expression of CD69 on T-lymphocytes. Therefore, in this study, CD25, CD64, and CD69 were used as cell surface biomarkers and their expression, as well as the population of positive cells, on leukocytes, neutrophils, monocytes and lymphocytes were investigated, respectively. Based on results with clinical samples, a panel of combined biomarkers CD25, CD64, and CD69 was constructed, and resulted in an excellent performance that is more accurate than the use of each single parameter alone.

2. Material and methods

2.1. Septic and healthy blood sample collection

The study of clinical patient blood samples was reviewed and approved by the Texas Tech University Health Science Center Institutional Review Board in accordance with pertinent laws governing human subjects research. This project included 40 septic patients and 10 healthy volunteers, with 80 septic patient blood samples and 10 healthy blood samples. Each healthy volunteer provided one draw of blood sample, and each septic patient provided two draws. Informed consent was obtained from all septic patients and healthy volunteers. All septic patients were receiving antibiotic treatment in the Intensive Care Unit (ICU). The 1st patient blood sample was collected within 24 h after sepsis validation (limited by logistical demands of the hospital and our IRB protocol), and the 2nd draw was collected within 48 h after diagnosis. Antibiotic treatment was employed on each patient between the 1st and the 2nd blood draw. All blood samples were collected from the University Medical Center (UMC) in Lubbock, TX. qSOFA (quick Sepsis Related Organ Failure Assessment) scores were used to identify septic patients. Each infected patient that scored a two or three was diagnosed as septic. Healthy volunteers involved in this project were not receiving any acute or chronic medical treatment and were not taking any medications besides hormonal contraception. Both healthy and septic blood samples were stored in 4 mL BD Vacutainer™ Plastic Blood Collection Tubes with K_2EDTA (Fisher Scientific) at 4 °C until analysis.

2.2. Septic and healthy blood sample preparation

The blood sample was first shaken to achieve a homogenized distribution, and to reach room temperature. 400 µL of whole blood from each blood sample was separated evenly into 4 1.5 mL-centrifuge tubes. Each aliquot of 100 µL whole blood sample was lysed by mixing the sample with 900 µL of deionized water for exactly 30 s to remove erythrocytes. Immediately afterward, 110 µL of concentrated saline buffer was added to the lysed blood to restore osmolarity. The mixture was then centrifuged at 4500 rpm for 5 min to separate the erythrocytes and leukocytes. The supernatant was then discarded, and the leukocytes were resuspended with Phosphate-Buffered Saline (PBS) to the original volume (100 µL).

2.3. Flow cytometry analysis

Flow cytometry (FACSCalibur, Becton Dicknson) was used for the analysis of blood samples in this study. Four aliquots of 100 µL lysed blood were used for each single experiment: three of them were stained for CD25, CD64, and CD69 analysis, and the remaining aliquot was unstained as an instrument control. Biotin-antiCD25, biotin-antiCD64, and biotin-antiCD69 were added to corresponding blood samples. All samples were incubated at room temperature for 20 min. All stained blood samples were then washed with PBS buffer 3 times, with centrifugation at 4500 rpm for 5 min between each wash. After washing, each sample was resuspended with PBS buffer to 100 µL. 1 µL of allophycocyanin (APC)-streptavidin (1 mg/mL, Invitrogen) was added to each stained blood sample for fluorophore labeling and incubated a light-protected cabinet at room temperature for 20 min in the dark. Excess APC-streptavidin was removed by centrifugation at 4500 rpm for 5 min. PBS buffer was then added to each sample for a final volume of 500 µL. The detector voltage was adjusted using the unstained control. The relative cell surface antigen expression was determined using the geometric mean of APC fluorescence intensity.

3. Results and discussion

3.1. Clinical samples

40 patients who were undergoing treatment in the Intensive Care Units (ICU) were enrolled in this study. All patients were identified with sepsis by qSOFA (quick Sepsis Related Organ Failure Assessment) criteria. Further SOFA (Sepsis Related Organ Failure Assessment) scores were calculated and vital signs were recorded for the specific antibiotic treatment for each septic individual. All vital signs of studied patients were listed in Table 1 (data from both genders is pooled, as there was no statistical difference between genders).

3.2. CD64 expression and CD64 + population

A significant difference in leukocyte CD64 expression between healthy volunteers and the 1st blood draw of septic patients was observed ($p = 0.013$, 95% confidence interval, Fig. 1). For further investigation, different subtypes of leukocyte, including neutrophil, monocyte, and lymphocyte, were analyzed separately using scatter gating (Fig. S1). Among all septic patients, high CD64 expression was observed on neutrophils and monocytes, while it remained low on lymphocytes (Fig. 2).

Both neutrophil CD64 expression and monocyte CD64 expression significantly increased in septic patients ($p = 0.004$ and $p = 0.002$ for the 1st blood draw, respectively; 95% confidence interval), while lymphocyte CD64 expression did not show strong difference ($p > 0.05$

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