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GPR18 expression on PMNs as biomarker for outcome in patient with sepsis

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ABSTRACT

Aims: GPR18, a G protein-coupled receptor (GPCR), is involved in bacterial clearance and survival in microbial sepsis. In this study, we examine GPR18 expression on polymorphonuclear neutrophils (PMNs) of patients with sepsis and to determine the potential association with disease severity and outcomes.

Main methods: We enrolled 81 patients admitted at the intensive care unit (ICU) with the diagnosis of sepsis. PMNs GPR18 expression was measured by flow cytometry at admission. Sequential Organ Failure Assessment (SOFA) and Acute Physiology and Chronic Health Evaluation (APACHEII) as well as other biomarker were measured at admission. Cox regression analysis was used to determine the influence of PMNs GPR18 expression on 28-day mortality.

Key findings: Patients with sepsis had a decreased percentage of PMNs bearing GPR18 in comparison with healthy subjects ($P < 0.001$). Compared with survivors, non-survivors had lower percentage of GPR18-positive PMNs, but higher SOFA, APACHEIIscores, and WBC count. There were inverse correlations between the percentage of GPR18-positive PMNs and APACHEII, SOFA score and C-recreative protein (CRP). Using Kaplan-Meier analysis, high percentage of PMNs expressing GPR18 (≥43.7%) was associated with a preferable 28-day survival ($P = 0.004$). High percentage of PMNs expressing GPR18 (≥43.7%) was significantly and independently associated with 28-day mortality, with a hazard ratio of 0.36 ($P = 0.37$). Moreover, LPS-Toll-like receptor (TLR)4 signaling mediated the GPR18 expression on PMNs.

Significance: These results indicate that decreased percentage GPR18-positive PMNs is associated with increased severity and poorer outcome of sepsis.

1. Introduction

Sepsis is a major cause of mortality in the ICU, and the mortality rate varies between 30% and 60% [1]. Early diagnosis and appropriate antibiotic treatment can have an important impact on the septic patient outcome. Therefore, much effort had been directed to determine biomarkers that can identify the sepsis and access disease severity and outcomes.

Sepsis is characterized by a progressive, injurious, generalized excessive inflammatory responses associated with multiple organ dysfunction [2]. Neutrophils are a crucial component of the innate immune response and the first leukocytes to be recruited to the inflammatory site against invading pathogens. Previous study has demonstrated that neutrophil function is substantially dysregulated during severe sepsis, resulting in marked defect in recruitment of neutrophils to infectious sites and inadequate antimicrobial responses [3]. At the same time, neutrophils that accumulate deleteriously in healthy tissue could release lytic enzyme and lead to tissue damage and multiple-organ failure [4]. These impaired migration and alterations in neutrophil functions could result from dysregulated neutrophil G protein-coupled receptor (GPCR) expression and downstream signaling in sepsis [5]. Recent studies have identified that GPR18 plays a vital role in activating the signaling pathways that control infectious inflammation and promote organ protection [6,7]. GPR18 has been detected on the surface of human leukocytes, including monocytes, PMNs and macrophages [6]. Using genetic manipulation of GPR18 in both cells and animals, it has demonstrated that GPR18 expression contributes to enhanced human macrophage phagocytosis of *Escherichia coli* and ingestion of apoptotic PMNs induced by resolvin (Rv)D2, which is a potent endogenous pro-resolving lipid mediator in inflammation. In addition, they found that

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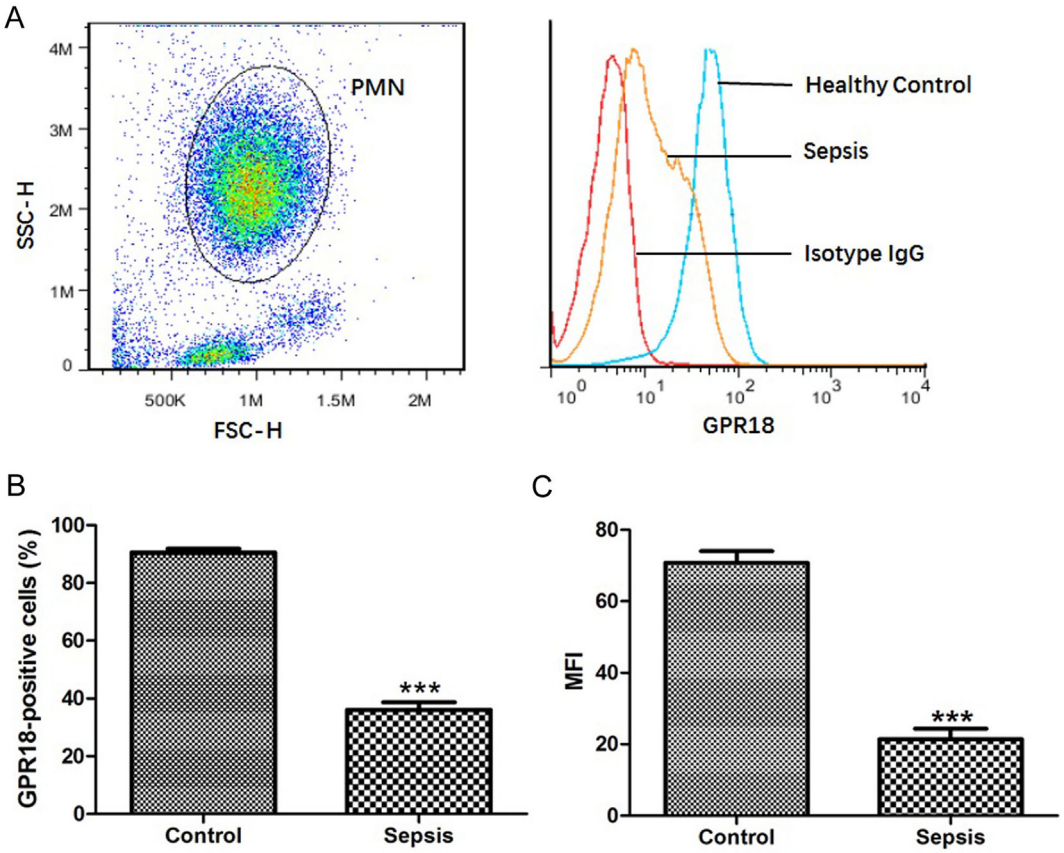

Fig. 1. Flow cytometry of GPR18 expression on PMNs. (A) Human whole blood from septic patients or healthy subjects were incubated with rabbit anti-GPR18 IgG or rabbit IgG (Isotype IgG), followed by Alexa Fluor® 488-anti–rabbit IgG. Panel A is a representative flow cytometry plot for panel B and C. (B) The percentage of GPR18-positive PMNs were significantly lower in patients with sepsis compared to healthy control subjects. Data are represented as mean percentage of PMNs ± SEM. ***$P < 0.001$. (C) The MFIs of GPR18 on PMNs were lower in patients with sepsis when compared with healthy control subjects. Data are represented as mean MFI ± SEM. ***$P < 0.001$.

GPR18-deficient mice showed excessive PMN infiltration and decreased PMN phagocytosis of *Escherichia coli* [6]. These finding indicated that GPR18 mediated multiple important functions involved with PMNs. Given alteration in neutrophils function are directly correlated with increased morbidity and mortality mediated by sepsis [8,9], we hypothesized that PMN GPR18 expression may be a potential biomarker of patients with sepsis.

The purpose of the present work is to determine whether there is differential expression of GPR18 on PMNs from patients with sepsis when compared with healthy control subjects. Also, we will assess the GPR18 expression on PMNs in the evaluation of the sepsis severity and outcome and the relationship between GPR18 expression and other biomarkers.

2. Materials and methods

2.1. Patients and controls

The study included 81 patients with sepsis depending on the definitions of the Sepsis Survival Campaign 2012 [10]. Exclusion criteria were pregnancy, age < 18 years, human immunodeficiency virus infection, corticosteroids treatment or immunosuppressive treatment in the previous six months, and cardiac shock. Thirty healthy individuals with no evidence of disease served as controls. Blood samples were collected within 24 h of ICU admission and white blood cell (WBC) count and CRP were recorded. Both APACHEII score [11] and SOFA score [12] were calculated at admission. The calculation of APACHE based on age, initial values of 12 routine physiologic measurements and previous health status. The score of APACHEII, Acute physiology and chronic health evaluation system, can be used to evaluate the severity of disease. SOFA is short for Sequential Organ Failure Assessment. The purpose of SOFA score is to describe the occurrence, development and evaluation of multiple organ dysfunction syndrome (MODS). We calculate Demographic, clinical, primary site of infection, biochemical, and microorganism data were obtained throughout the ICU stay. The study protocol was approved by the Tianjin Nankai Hospital Ethics and Medical Research Committee which waived informed consent, because the study was observational and residual blood after completing routine follow-up period was used to detect GPR18 expression.

2.2. Human PMN isolation

Human PMNs were isolated from heparinized venous blood drawn from healthy volunteers, who denied taking any medication at least two weeks before donation. PMNs were purified using Polymorphprep™ (AXIS-SHIELD, OSLO, Norway). PMNs purity was routinely 94–98%. Replicate experiments used PMNs from different volunteers.

2.3. Drug treatments

To investigate whether LPS-TLR4 signaling is involved in regulating the expression of GPR18 on PMNs, isolated human PMNs from healthy subjects and patients with sepsis were treated with LPS (TLR4 agonist) alone with different concentration or pretreated with TAK-242 (TLR4 antagonist), then treated with LPS. TAK-242 selectively inhibits TLR4 by binding to the intracellular Cys747 residue and disrupts its

Table 1
General characteristics of the patients.

	Healthy controls (n = 30)	Sepsis patients (n = 81)
Age (years)	54.5 ± 3.1	61.3 ± 1.8
Gender male (n, %)	11(36.7)	62(76.5)
Comorbidities (%)		
Chronic heart disease		36.8
Diabetes		17.1
Chronic respiratory disease		19.7
Chronic kidney disease		5.0
Digestive/liver disease		21.1
Other		2.6
Diagnostic category (%)		
Cardiovascular		2.6
Respiratory		19.7
Abdominal		56.6
Urinary		2.6
Neurologic		5.3
Unknown		3.9
WBC ($\times 10^3$/μl)		14.53 ± 0.91
CRP (μg/ml)		155.2 ± 12.79
GPR18 (positive cells %)	91.4 (86.6–96.1)	33.13 (20.42–47.5)
Serum lactate (mmol/L)		2.37 ± 0.28
SOFA score		8 (1 − 21)
APACHE II score		16 (3–44)
Mechanical ventilation (%)		51.9

Results are expressed as the mean ± SEM or median (interquartile range). WBC = white blood cell; CRP = C-reactive protein; SOFA = Sequential Organ Failure Assessment; APACHE II = Acute Physiology and Chronic Health Evaluation.

interaction with adaptor molecules TIRAP and TRAM.

2.4. Flow cytometric analysis of GPR18 expression on PMN surface

For each analysis, peripheral blood was collected in evacuated tubes containing with ethylenediamine tetraacetic acid (EDTA) as the anticoagulant. 50 μl of whole packed blood was incubated with 10% donkey serum for 10 min. Cells were fixed with 2% paraformaldehyde (PFA) for 10 min and permeabilized with 0.1% PBS-Triton X-100 for 10 min. Then, each sample was incubated with rabbit anti-human GPR18 (1: 250, Abcam, Cambridge, UK) for 30 min at room temperature, followed by Alexa Fluor® 488-donkey anti–rabbit IgG (1:2000, Abcam) in the dark for 30 min. Rabbit polyclonal IgG served as isotype control (1: 250, Abcam). Whole blood sample need a RBC lysis step with Human Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN, USA). GPR18 expression on PMNs were monitored by FACSCalibur equipped with CellQuest software (BD Bioscience, San Diego, CA, USA). PMNs were distinguished from other leucocytes and platelets by their characteristic forward and side-scatter distributions (Fig. 1A). Results were obtained within 3 h of blood collection and displayed on single parameter histograms of FITC fluorescence ($\log_{10}$ scale). The cursor was set so that no > 1% of the cells in the negative control sample stained positively with the isotype control antibody. Results were presented as either the percentage of cells bearing GPR18 or as the mean fluorescence intensity (MFI).

2.5. Statistical analysis

Continuous variables were presented as mean ± standard error of the mean (SEM), or median (interquartile range, IQR). Categorical variables were reported as number or percentage. Continuous variables were compared by Student's unpaired t-test or the Mann-Whitney U test (for nonparametric data). Categorical variables were compared by the chi-squared test or Fisher's exert test. The Person correlation coefficient or Spearman's non-parametric correlation coefficient was used to calculate the association between GPR18 and other investigated biomarker and severity scores. Receiver operating characteristics (ROC) curves were used for GPR18 to discriminate between survivors and non-survivors at 28 days, and the areas under the ROC curve (AUC) were determined. The univariate Cox analysis was used to assess the prognostic value of factors for 28-day mortality. We also performed a multivariate Cox regression analysis to determine those factors independently associated with mortality. Variables that were significant ($P < 0.05$) after univariate analysis were entered into the multivariate analysis. All statistical tests were two-sided and a P value < 0.05 was considered statistically significant. We used IBM SPSS statistics 19.0 (SPSS Inc., Chicago, IL) to perform statistical analysis.

3. Results

3.1. GPR18 expression in human PMNs

PMN GPR18 expression was measured within 24 h of ICU admission in 81 patients. Fig. 1A shows the GPR18 expression of human PMNs in the whole blood of patients with sepsis and the healthy control. There were far less GPR18-positive PMNs in patients with sepsis than in healthy control (mean 36.12 ± 2.62% versus 90.64 ± 1.27%, $P < 0.001$) (Fig. 1B). The GPR18 expression on PMNs was also significantly lower in septic patients compared to healthy controls when the results are presented as the MFI (mean 70.74 ± 3.29 versus 21.42 ± 2.94, $P < 0.001$) (Fig.1C). We also analyzed the GPR18 expression on both monocytes and lymphocytes in the whole blood. Compared with healthy controls, patients with sepsis had a decreased percentage of monocytes bearing GPR18 (mean 4.22 ± 0.86% versus 13.72 ± 1.74%, $P < 0.001$) (Supplementary Fig. 1). However, lymphocytes did not express GPR18 (Supplementary Fig. 1A).

The demographic data of the healthy controls and patients are shown in Table 1. Among these 81 patients with sepsis, 59 showed positive microbiology cultures and 22 patients presented a strong clinical suspicion for infection. Table 2 showed the corresponding microorganism.

The baseline characteristics of patients with sepsis are summarized in Table 3. There are 37 patients who died among these investigated patients. At ICU admission, the percentage of PMNs bearing GPR18 was

Table 2
Microorganism isolated from patients with sepsis.

Gram-positive (n, %)	
Staphylococcus aureus	1(1.3)
Enterococcus faecium	10(12.5)
Staphylococcus epidermidis	6(7.5)
Enterococcus casselifavus	2(2.5)
Staphylococcus hominis	2(2.5)
Other *Staphylococci*	4(5)
Viridans Streptococci	5(6.3)
Kocuria roseus	1(1.3)
Stretococcus anginocus	1(1.3)
Corynebacterium striatum	1(1.3)
Gram-negative	
Klebsiella pneumoniae	6(7.5)
Escherichia coli	13(16.3)
Aeromonas species	1(1.3)
Acinetobacter baumannii	5(6.3)
Enterobacter cloacae	3(3.8)
Neisseria species	2(2.5)
Enterobacter aerogenes	2(2.5)
Burkholderia cepacia	1(1.3)
Proteus vulgaris	1(1.3)
Klebsiella oxytoca	1(1.3)
Stenotrophomonas maltophilia	1(1.3)
Serratia marcescens	1(1.3)
Fungi	
Candida albicans	3(3.8)
Candida tropicalis	1(1.3)

Table 3
Comparisons of characteristic between survivors and non-survivors of sepsis.

Variables	Survivors (n = 44)	Nonsurvivors (n = 37)	P value
Age (years)	63.2 ± 2.2	62.0 ± 2.6	0.737
Gender male, n (%)	31 (70.5)	31 (83.7)	0.158
Site of infection, no. (%)			0.490
Respiratory	9 (20.5)	12(32.4)	
Abdominal	30(68.2)	22(59.5)	
Urinary	2(4.5)	0(0)	
Cardiovascular	2(4.5)	2(5.4)	
Unknown	3(6.8)	3(8.1)	
Bacteremia (%)	5(11.4)	10(27.0)	0.071
Septic shock (%)	6 (13.6)	10 (27.0)	0.132
WBC ($\times 10^3$/μl)	12.53 ± 1.04	16.98 ± 150	0.014
SOFA score	7 (4–9)	10 (7–12)	0.001
APACHE II score	10 (3−30)	23 (11–44)	< 0.001
CRP (μg/ml)	130.5 ± 14.07	151.8 ± 18.52	0.356
GPR18 (positive cells %)	43.45 ± 3.59	27.52 ± 2.95	0.001
Serum Lactate (mg/dl)	2.03 ± 0.19	2.72 ± 0.51	0.181
Mechanical ventilation, no. (%)	21 (47.7)	23 (62.2)	0.194

Results are expressed as the mean ± SEM or median (interquartile range). WBC = white blood cell; CRP = C-reactive protein; SOFA = Sequential Organ Failure Assessment; APACHE II = Acute Physiology and Chronic Health Evaluation.

significantly lower in non-survivors than in survivors (mean 26.94 ± 4.15 versus 44.99 ± 4.33; P = 0.005). In addition, compared with survivors, non-survivors had higher SOFA, APACHEIIscores, and WBC count, whereas the CRP concentration did not differ significantly.

3.2. Relationship between GPR18 and indices of inflammation and infection

High concentration of CRP and increased WBC count are considered as biomarker of inflammation and infection. In the present study, there was a significant correlation between GPR18 expression and plasma level of CRP (r = −0.42, P = 0.001) but not between GPR18 expression and WBC count (Fig. 2).

3.3. Association between GPR18, mortality, and severity of sepsis

To determine the optimal cutoff values of percentage of PMNs bearing GPR18 for 28-day mortality, we performed ROC analysis. The AUC which relate percentage of PMNs bearing GPR18 to survival was 0.71 (P = 0.007) (Fig. 4 A). The optimal cutoff value was 43.7%, resulting in a sensitivity of 83%, specificity 49%, PPV of 56%, and NPV 80%. Patients with high percentage of PMNs bearing GPR18 (≥43.7%) had a better survival, according to Kapan-Meier survival curves (P = 0.004) (Fig. 4B). Cox regression analysis showed that percentage of PMNs bearing GPR18 (≥43.7%) was significantly and independently associated with 28-day mortality, with a hazard ratio of 0.36 (95%CI, 0.13–0.94; P = 0.037; Supplementary Table 1).

The percentage of GPR18-positive PMNs was negatively correlated with the SOFA score (r = −0.31, P = 0.006) and APACHEIIscore (r = −0.27, P = 0.03) (Fig. 3), suggesting that the percentage of PMNs bearing GPR18 was associated with the severity of sepsis. The diagnostic accuracy of SOFA and APACHEIIscores for predicting 28-day mortality was also evaluated by AUC, sensitivity, specificity, PPV, and NPV (Supplementary Table 2). When the GPR18 expression and SOFA or APACHEIIscore were combined, both the PPV and NPV were improved compared to GPR18, SOFA, or APACHEIIalone (Supplementary Table 2).

3.4. GPR18 expression on PMNs was dependent on LPS

Previous studies have demonstrated that LPS-TLR4 signaling pathway is involved in the pathophysiology of polymicrobial sepsis [13,14]. In addition, the expression of CCR2, a GPCR, can be regulated by the direct activation of TLR4 with LPS [15]. Therefore, we try to determine whether LPS-TLR4 signaling is involved in regulating the expression of GPR18 on PMNs. We isolated human PMNs from healthy individuals and incubated them with 0.l, 1, 10, and 20 μg/ml of LPS for 30 min. Flow cytometry revealed that the LPS treatment decreased the percentage of GPR18-positive PMNs in a dose-dependent manner compared with control cells (P < 0.001) (Fig. 5A). We next confirmed that these changes in GPR18 expression were not due to the changes in cellular viability, because the LPS-treated PMNs and the control cells had similar percentage of cell viability (Annexin V^-/PI^-) (Supplementary Fig. 2). In addition, we found a time-dependent reduction in percentage of GPR18-positive PMNs treated with 10 μg/ml of LPS (P < 0.001) (Fig. 5B). Using selective Toll-like receptor 4 (TLR4) antagonist TAK-242, we confirmed the effect of LPS on the surface GPR18 expression of PMN through TLR4. As shown in Fig. 5C, TAK-242 completely blocked the reduction of GPR18 expression on PMN induced by 10 μg/ml of LPS treatment (P < 0.05).

We also isolated PMNs from septic patients and incubated them with 0.l, 1, and 10 μg/ml of LPS for 30 min. The results showed that LPS treatment decreased the percentage of PMNs bearing GPR18 (P < 0.05) (Supplementary Fig. 3).

4. Discussion

The present study investigated the GPR18 expression on PMNs in patients with sepsis and healthy subjects. We found that the percentage of PMNs bearing GPR18 was significantly lower in patients with sepsis compared with healthy individuals and the percentage of PMNs bearing GPR18 was associated with the severity of sepsis and patient prognosis.

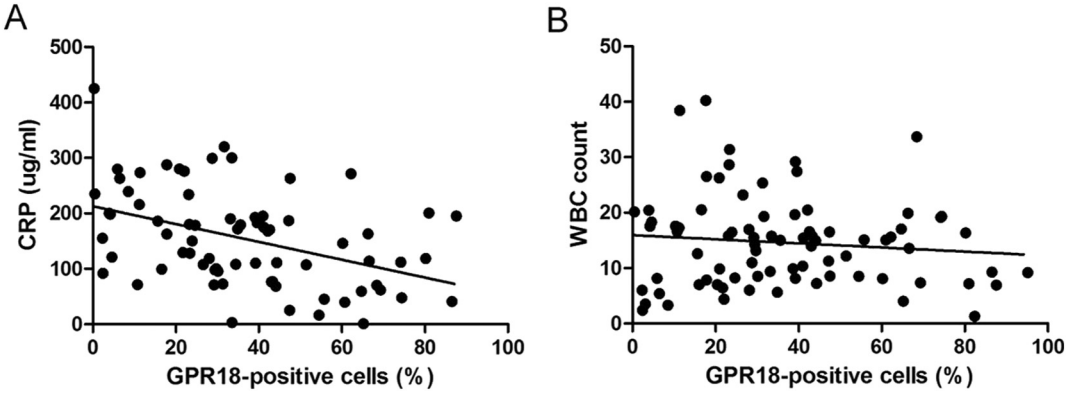

Fig. 2. Relationship between the distribution of GPR18-positive PMNs with CRP levels and WBC count. (A) There was an inverse correlation between the percentage of GPR18-positive cells and CRP levels (r = −0.42, P = 0.001). (B) There were no relationships between the percentage of GPR18-positive cells and WBC count.

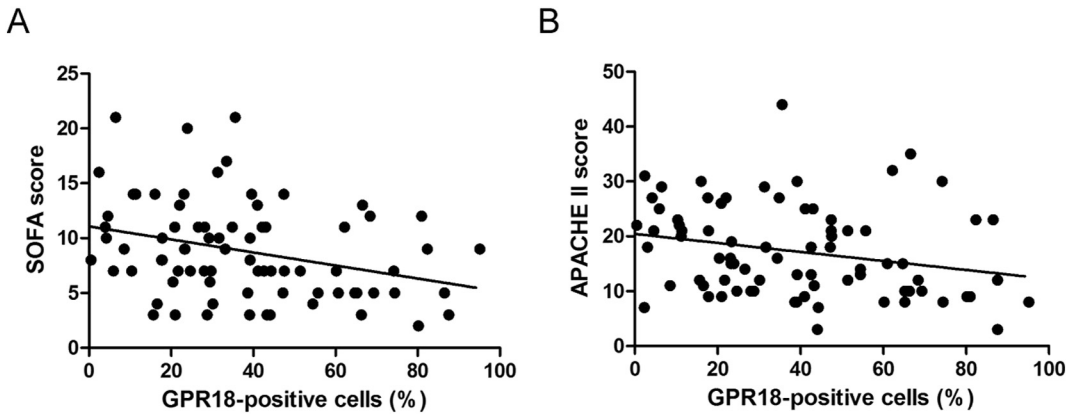

Fig. 3. Association of the distribution of GPR18-positive PMNs with SOFA score and APACHEIIscore. (A) The percentage of GPR18-positive cells was negatively correlated with SOFA score ($r = -0.31$, $P = 0.006$). (B) The percentage of GPR18-positive cells was negatively correlated with APACHEIIscore ($r = -0.27$, $P = 0.03$).

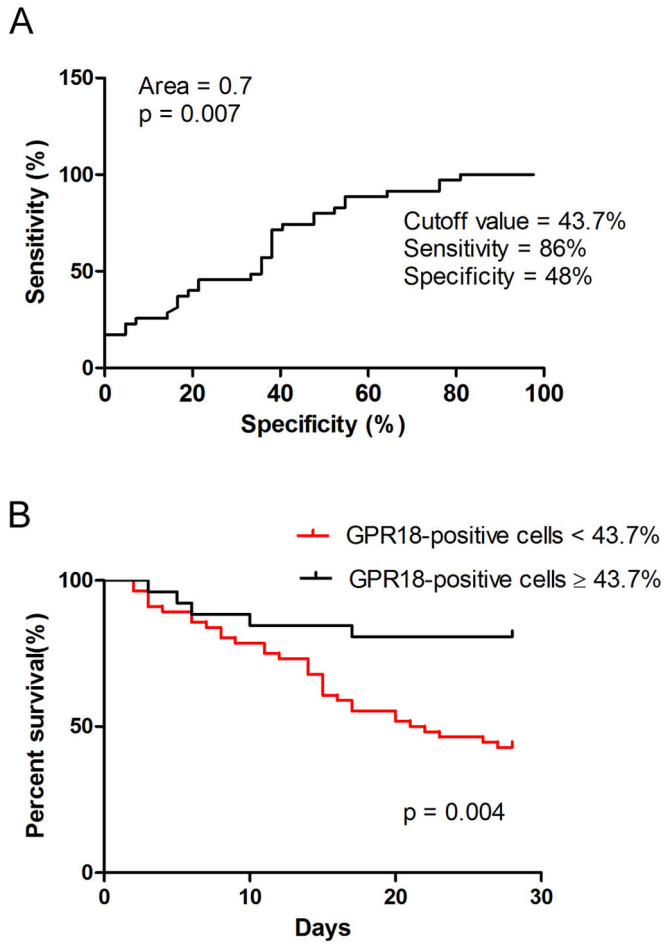

Fig. 4. ROC curve and Kaplan-Meier curve analysis for 28-day mortality. (A) ROC curve determines the cutoff value of percentage of GPR18-positive PMNs to discriminate between survivors and non-survivors. (B). Kaplan-Meier curves of 28-day survival with patients grouped according to the percentage of GPR18-positive PMNs ≥ 43.7% or < 43.7%. Log-rank test was used to assess the difference between groups.

Furthermore, we found that LPS-TLR4 signaling mediated the GPR18 expression on PMNs.

Although GPR18 localization is less studied in humans, but it has been found that GPR18 is ubiquitously expressed across a wide range of tissues [16], with higher expression in the testis [17], the brain [18], the cells of immune system [19,20], and the endometrial cells [21]. So far, the physiological function of GPR18 remains largely unclear, but several studies indicated possible roles for this GPCR. For example, McHugh et al. demonstrated GPR18 expressed on microglial cells of brain regulates cell migration after central nervous system injury or inflammation [22]. Penumrti and Rahman showed that GPR18 located in rosttal ventrolateral medulla is involved in controlling central blood pressure in rat [18]. Recently, GPR18 was identified as a specific GPCR mediating pro-resolving actions of RvD2 with phagocytes. RvD2 derived from docosahexaenoic acid (DHA) is a potent immunoresolvent biosynthesized during the resolution phase that limited acute inflammation in vivo [23]. Endogenous RvD2 is produced in human neutrophils, macrophage, adipose tissue, breast milk, lung, and sepsis patients. In human macrophages, RvD2-GPR18 interaction stimulates phagocytosis of microbes and apoptotic PMNs to accelerate resolution of bacterial infections and organ protection [6]. In addition, it was found that RvD2 stimulated PMNs phagocytosis of microbes by increasing intracellular reactive oxygen species generation [24], and recent study with GPR18-deficient mice further demonstrated that GPR18 mediated this action of RvD2 [6]. In animal model of disease, it is identified that RvD2 protect mice from death depending on the GPR18 receptor [7]. Taken together, these results indicated that GPR18 plays a vital role in mediated microbial killing, survival, and organ protection. Consistent with these findings, we found the distribution of blood PMNs bearing GPR18 was shown to be far lower in patients with sepsis than in healthy controls, and patients who survived have higher percentage of PMNs bearing GPR18 compared with those who died. Besides binding to RvD2, GPR18 could bind with some endogenous cannabinoids, such as *N*-arachidonoyl glycine (NAGly), abnormal cannabidiol (Abn CBD) and $\triangle^9$-tetrahydrocannabinol (THC). It has been indicated that endocannabinoids mediate the migration of neutrophils to the site of infection, and GPR55, a novel cannabinoid receptor, enhances the neutrophils responses to cannabinoid while inhibiting exaggerated tissue injury mediated by degranulation and respiratory burst [25]. In the future study, we will examine whether GPR18 is involved in the regulation of migration of neutrophil induced by cannabinoids.

In this study, we also observed there was a significant correlation between the percentage of GPR18-positive PMNs and the plasma levels of CRP but not between GPR18 and WBC count. Given CRP is commonly incorporated in routine diagnostic algorithms for inflammation and sepsis. Our results suggested that percentage of GPR18-positive PMNs could be considered as an inflammation and sepsis marker. The explanation of lack of correlation between GPR18 and WBC count could be due to the different kinetics of these two biomarkers, and the influence of age and sex on WBC count, but also the behavior of immune system. It is well established that WBC count is not always increased in

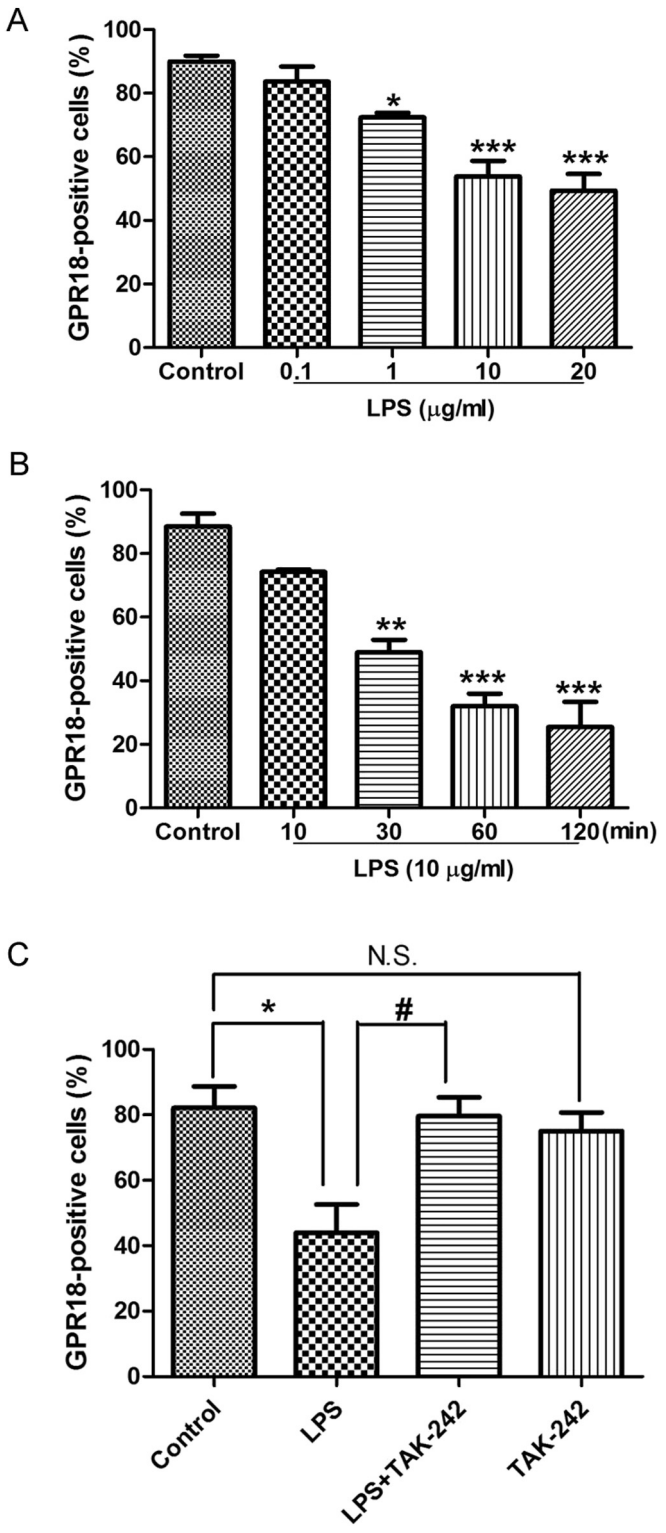

Fig. 5. LPS-TLR4 signaling mediated the GPR18 expression on PMNs. Flow cytometry of surface expression of GPR18 on PMNs isolated from healthy control subjects. (A) PMNs were incubated with LPS (0.1, 1, 10, and 20 μg/ml), or medium alone (Control) for 30 min. (B) PMNs were incubated with 10 μg/ml of LPS for different time, as indicated. (C) PMNs were pretreated with TAK-242 (1 μM) for 30 min and followed by 10 μg/ml of LPS stimulation for 30 min. Data are represented as mean percentage of PMNs ± SEM. $^{*}P < 0.05$ relative to control group, $\#P < 0.05$ relative to LPS group. $^{***}P < 0.001$ relative to control group.

severe septic patients, because of the immunosuppressive state induced by the severe septic syndromes.

SOFA score and APACHEIIscore are well-established markers to predict outcomes of patients with severity of sepsis [11,12]. Consistently, our findings showed that patients who survived had lower SOFA scores and APACHEIIscores compared to those who died. Moreover, we observed that both SOFA and APACHEIIscore were negatively correlated with the numbers of GPR18-positive PMNs. These results suggest that the percentage of PMNs bearing GPR18 is associated with the severity of sepsis. SOFA score also acts as an indicator of the extent of organ injury [26]. Given the inverse correlation between the SOFA score and the numbers of GPR18-positive PMNs, our result suggested that GPR18 expression on PMNs contributes to organ protection during sepsis. Using GPR18-deficient mice, it has demonstrated that the protective actions of RvD2 depend on the GPR18 receptor during PMNs mediated lung injury [6]. Thus, our clinic data are in line with this animal study. With Kaplan-Meier survival-curve analysis, it was revealed that patient with high percentage of GPR18-positive PMNs (≥43.7%) survived better than those with low percentage. Furthermore, the percentage of PMNs bearing GPR18 (≥43.7%) was significantly and independently associated with 28-day mortality in the multivariate Cox regression analysis. These findings demonstrated that the GPR18 expression on PMNs could act as a biomarker to evaluate the outcome of sepsis.

Neutrophils are generally considered to be a homogenous population of terminally differentiated cells, whose functions are restricted to phagocyte and kill pathogen. However, increase evidence has demonstrated the existence of different sub-populations of neutrophils with distinct phenotypic and functional activity during sepsis. For example, in a human model of LPS challenge, based on the expression of CD16 and CD62L, three different subsets of inflammatory neutrophils were discriminated: $CD16^{high}/CD62L^{high}$, $CD16^{high}/CD62L^{low}$, and $CD16^{low}/CD62L^{high}$. The $CD16^{high}/CD62L^{low}$, and $CD16^{low}/CD62L^{high}$ cells were not found in healthy donors [27]. Moreover, other researches also demonstrated that circulating neutrophils presented with different phenotypic and functional subsets after sepsis onset [28,29]. Given different subsets of neutrophils with distinct phenotype, we hypothesized the change degree in GPR18 expression on each sunset was in distinct level during sepsis. In the present study, we take the neutrophils as a homogenous population to analyze the GPR18 expression. Our results showed the weak correlations between GPR18 expression and disease severity, and 28-day mortality. In the future study, we should firstly determine the GPR18 expression on each subset of neutrophils after sepsis onset, then analyze the association of GPR18 expression of subsets with other biomarker or predictor for survival. Under this condition, GPR18 expression on subsets of neutrophils will be act as a more useful biomarker for the severity and outcome prognosis in patients with sepsis. The diagnostic accuracy of SOFA and APACHEIIscores to predict mortality was also evaluated. When the GPR18 expression and SOFA or APACHEIIscore were combined, both the PPV and NPV were improved compared to GPR18, SOFA, or APACHEIIalone.

GPR18 expression has been detected on the surface of PMNs from human peripheral blood. However, the mechanism underlying the regulation of GPR18 expression on PMNs has not yet been investigated. A growing body of evidence indicates that TLRs contribute significantly to the overwhelming inflammatory response and multiple organ injury during sepsis [13,30]. Specifically, the study using mice indicated the importance of TLR4 in the pathophysiology of polymicrobial sepsis [14]. In addition, both in vitro and in vivo experiments have demonstrated that direct activation of TLR4 with LPS is to able to regulate the chemokine receptor CCR2, a GPCR, expression on neutrophils [15]. Moreover, our finding demonstrated that decreased frequency of GPR18-positive PMNs in sepsis patients is correlated with organ injury and severity of sepsis. Therefore, we hypothesize that LPS-TLR4 signaling may regulate the expression of GPR18 on PMNs during sepsis. The results showed that LPS treatment reduced the percentage of

GPR18-positive PMNs in a concentration and time-dependent manner. In addition, we found that pretreatment of selective TLR4 antagonist TAK-242 prevented the down-regulation of GPR18 on human circulating PMNs. The major regulatory mechanism of GPCR is desensitization and internalization [31]. G protein-coupled receptor kinases 2 (GRK2) are serine/threonine protein kinases that play a prominent role in regulating receptor phosphorylation and internalization of almost all GPCR families [32]. Moreover, markedly increased expression of GRK2 has been found in neutrophils from septic patients [33]. Therefore, in the future, we will investigate whether the TLR4 activation down-regulate the GPR18 expression on PMNs during sepsis by increasing GRK2 expression.

To our knowledge, this is the first study to assess the GPR18 expression on PMNs of patients with sepsis. We found that GPR18 expression on PMNs associated with organ protection during sepsis. Compelling evidence has indicated that impaired direction migration of neutrophils occurs after severe sepsis. Under this condition, neutrophils cannot reach infection sites due to the inability to follow the guidance of chemokine gradients, and antimicrobial ability of PMNs becomes useless. Simultaneously, neutrophils veer away from the guidance of chemical gradients, and deleteriously accumulate within remote vital organ, leading to tissue damage and ultimately organ dysfunction [4] . Recently, GPR18 has been indicated to promote cell direct migration in response to *N*-arachidonoyl glycine [34], an efficacious agonist at GPR18. Moreover, RvD2 treatment restored the defective directional migration of neutrophils and improved survival after burn injury [35]. Thus, these findings together our results indicated that GPR18 expressions on PMNs may promote organ protection by control of the directional migration of PMNs.

There are several limitations in our study. First, the sample size of this study is small, preventing subgroup analysis to examine the effects of type of admission, comorbidities or infection site on the GPR18 expression in PMNs. Second, this study was conducted in a single medical center, limiting the generalizability of results to setting with various cases. Third, we did not perform a serial measurement for GPR18 expression on PMNs and SOFA score. The aim of this study was to investigate whether the GPR18 expression on PMNs could act as a biomarker accessing severity and mortality early in septic patients. Therefore, we measured the GPR18 expression on PMNs within 24 h instead of serial measurement. Further sequential study is needed to determine if changes in the percentage of GPR18-positive PMNs are associated with SOFA score.

Considering the SOFA score and APACHEIIscore as predictors of sepsis severity, the correlation between the percentage of GPR18-positive PMNs and these scores make it possible to consider GPR18 expression as a potential biomarker of the severity of sepsis. Moreover, the percentage of PMNs bearing GPR18 was significantly and independently associated with 28-day mortality.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.11.061.

Conflict of interest

The authors have declared that no competing interests exist.

Author contributions

L. Zhang and C. Qiu contribute equally to this work. X.Wang devised the experimental design and wrote the paper. C.Qiu was responsible for the enrolment of patients with sepsis and healthy control subjects. L. Zhang and L. Yang performed the flow cytometry analysis of GPR18 expression on blood PMNs and the isolation of PMNs from human whole blood. L. Zhang, C. Qiu and Z. Zhang were involved in the acquisition and analysis of clinic data. Q. Zhang and B. Wang provided technical assistance and the presentation of the figures.

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