Identification of Circulating Tfh/Th Subsets as Biomarker of Hospital-acquired Pneumonia

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Research Article

Keywords: Hospital-acquired pneumonia, Tfh cell, Th, Prognosis, PCT

Posted Date: December 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1153884/v1

Abstract

Background

The incidence rate of hospital-acquired pneumonia (HAP) is increasing in ICU patients, which is usually associated with dysregulated immune responses. Previous study has revealed Follicular helper T (Tfh) cells were essential for the formation and maintenance of geminal centers for anti-viral immune response, however, little is known about it during HAP.

Methods

A total number of 62 patients with HAP and 10 healthy individuals were recruited. Lower respiratory tract secretion and blood samples were taken for microbiological examinations. Uncontrolled and controlled HAP patients were identified on the basis of its respiratory function or hemodynamics, according to the ATS guidelines of HAP. Circulating Tfh cells (CXCR5+Foxp3-CD4+) and Th cells (CXCR5-FoxP3-CD4+) in all individuals were analyzed by flow cytometry.

Results

Clinically, 34 patients had uncontrolled HAP and 28 patients were controlled HAP. Patients were mainly infected with Klebsiella pneumoniae (K.p), Acinetobacter baumannii, Escherichia coli, and Pseudomonas aeruginosa. It is noted that Tfh/Th ratio was increased in patients with uncontrolled HAP than controlled HAP (P<0.05). Especially, Tfh/Th ratio was also higher in K.p-infected than non-K.p-infected patients (P<0.05). Furthermore, Tfh/Th ratio was significantly elevated in patients with BSIs compared to those without BSIs (P<0.01). Furthermore, Tfh/Th ratio showed an association with PCT, and the combination of Tfh/Th and PCT could serve as a better predicting marker for deterioration of HAP. Accordingly, HAP patients with high Tfh/Th ratio had a lower rate of survival in 28 days.

Conclusion

Tfh/Th ratio is useful for identifying the severity of the patients with HAP and increased Tfh/Th ratio indicates uncontrolled HAP. Circulating Tfh/Th subsets could be used as a prognostic biomarker and may provide novel insight for the pathogenesis of HAP.

1. Introduction

In the intensive care unit (ICU), hospital-acquired pneumonia (HAP) remains as the most common infection [1]. Pneumonia is usually associated with dysregulated immune responses. The immunopathogenesis of pneumonia is very complex that still remains rather unclear. Resistance, or actions of the host to eradicate living microbes, in the lungs involves a combination of innate and adaptive immune responses triggered by air-space infection [2].

Follicular helper T (Tfh) cells are antigen-experienced T cells that can be found mainly in the B cell zone of secondary lymphoid organs and to a less extent in circulation [3]. Tfh cells are known to be closely related to viral infection like HIV, but their relation to bacterial infections has not been widely studied. Usually, Tfh cells are identified by their constitutive expression of CXCR5 and play an indispensable role in the formation and maintenance of germinal centers for ongoing immune responses [4]. Whether the Tfh cells could serve a role in bacterial pneumonia assessing and prognosis remains to be investigated.

In the present study, we investigated the frequency of Tfh cells and evaluated the ratio of Tfh/Th among subjects of HAP, which has been identified that the ratio of Tfh/Th could serve as a useful biomarker for management of HAP.

2. Material And Methods

2.1 Patients

A total number of 62 patients with HAP and 10 healthy individuals were recruited in our study. HAP was diagnosed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) standards. HAP patients with one or more of the following conditions were excluded from this study: 1) asthma; 2) autoimmune diseases; 3) chronic obstructive pulmonary disease (COPD) and malignant disease. All protocols involving human participants were reviewed by the Ethics Committee of the First People's Hospital of Kunshan Affiliated to Jiangsu University (Approval No.2019-112-2).

2.2 Definition

Patients with both of the following conditions were considered having uncontrolled pneumonia as previously described: 1) having two or more complications (e.g. septic shock, heart failure, acute respiratory distress and secondary infections) and 2) one or more of these complications did not improve after 3 days of active treatment [5, 6].

Blood stream infection (BSIs) were defined by positive blood culture or cultures with an isolate of the same species grown in at least one blood culture bottle in a patient with systemic signs of infection (i.e. a patient who has evidence of one or more of the symptoms or signs, which are fever (body temperature > 38 ◦C), hypothermia (body temperature < 36◦C), chills, hypotension, oliguria, or high lactate levels)[7].

2.3 Flow cytometry

Flow cytometry analysis of the cell subsets in the peripheral blood was performed as previously described with modifications [6]. In brief, 5 mL of venous blood was drawn from each patient into anti-coagulant tubes. Human peripheral blood cells were first stained with APC-anti-CD45 (BD, Catalog # HI30), FITC-anti-CD4 (BD, Catalog # 550628), PerCP-anti-CXCR5 (BD, Catalog # RF8B2) for 30 min in the dark at room temperature. Staining for Foxp3 with PE-anti-Foxp3 (BD, Catalog #259D/C7) was performed

after fixation and permeabilization of the cells. Data was acquired using a BD FACS Calibur cytometer and data analysis was performed using FlowJo 10.2 Software (Figure1).

2.4 Laboratory testing

Lower respiratory tract secretion samples and blood samples were collected from each patient for microbiological examination. Serum were collected as well for procalcitonin (PCT) measurement, PCT was measured using Xin Chanye PCT automated immunoassays. The analytical sensitivity of all assays was <0.25g/L, as previously described [6].

2.5 Statistical analysis

All data in this study were expressed as mean ± standard deviation (SD), and all statistical analyses were performed with SPSS version 19.0 (SPSS Inc.). Student's t test was used to compare the difference between two groups. Spearman's correlation analysis was used to assess the correlation between PCT and Tfh/Th. Receiver operating characteristic (ROC) curves were calculated to select the cut-off level of ratio of Tfh/Th and PCT value indicating exacerbation of HAP. Kaplan-Meier survival analysis was performed to estimate the survival. All tests were two sided and a *P* value less than 0.05 were considered statistically significant.

3. Results

3.1 Patient characteristics

A total number of 62 patients were recruited in our current study, with the baseline clinical characteristics listed in Table 1. Of the 62 recruited patients, 38 were male and 24 were female, with age ranging from 18 to 85 years old. In this cohort, 34 patients had progressive HAP, 28 had controlled HAP, 21 were K.p-positive infected HAP and 10 were complicated with BSI. In addition, we enrolled 10 healthy individuals from the volunteer team as a healthy control group.

3.2 The ratio of Tfh/Th is increased in uncontrolled HAP patients

Firstly, we analyzed if these cell subsets were different in controlled and uncontrolled HAP cases. As shown in Figure 2, Tfh cells were significantly increased in uncontrolled HAP (15.47%±5.49) compared to controlled HAP (11.83%±4.69, *P*=0.01) and heathy group (10.76%±4.76, *P*=0.02). Notably, the ratio of Tfh/Th performed a significant increase in progressive HAP (0.19±0.08) compared to disease-controlled HAP (0.13±0.06, *P*=0.01) and healthy group (0.12±0.05, *P*=0.02). These data indicated that the ratio of Tfh/Th is increased in progressive HAP patients and maybe useful for rapidly recognizing severity in HAP.

3.3 The ratio of Tfh/Th is elevated in K.p-infected HAP patients

The pathogenic bacteria were detected in 62 patients with HAP, of which twenty-one patients were Klebsiella pneumoniae (K.p)-infected and forty-one patients were non-K.p-infected

including Acinetobacter baumannii (n=11), Escherichia coli (n=4), Proteus mirabilis (n=2), Pseudomonas maltophilia (n=6), Pseudomonas aeruginosa (n=8), Pseudomonas cepacia (n=2), Staphylococcus aureus (n=6), Aspergillus(n=2). Next, we further calculated Tfh cells to decide if these cells were relatively increased or decreased in special pathogenic bacteria-infected patients. Interestingly, we found that Tfh cells and the ratio of Tfh/Th were both significantly elevated in K.p-infected (16.20% ±4.37, 0.20±0.06) compared to non-K.p-infected patients (12.44%±5.61, 0.15±0.08) with *P*=0.01 and *P*=0.02, respectively (Figure 3A). These data suggest that the immune response varied by different bacterial infections, which is related to the Tfh.

3.4 Blood stream infection in HAP induced the raise of the ratio of Tfh/Th

BSI is one of the most frequent lethal conditions that are managed in the ICU. We then investigated these different CD4+ cell subsets in HAP with BSIs and those without BSIs. As shown in Figure 3B, Tfh cells were significantly increased in HAP with BSIs compared with those without BSIs patients (16.20%±4.37 vs 12.98%± 4.82, *P*=0.01). Also, the ratio of Tfh/Th showed significantly increasing in patients with BSIs than those without BSIs (0.22±0.11 vs 0.15±0.06, *P*=0.01). These data showed that the immune response caused by bacteria entering the blood will further mediate a more serious Tfh/Th imbalance.

3.5 The correlation between PCT and the ratio of Tfh/Th

PCT, the precursor of calcitonin, as a marker for bacterial pneumonia, is usually low or untraceable in the circulation in healthy people, but can increase significantly in individuals with bacterial infection. PCT 0.25 μg/l is recommended as the threshold to evaluate whether antibiotics are used in patients with respiratory infection [8, 9]. We next determined whether the ratio of Tfh/Th was correlated to PCT in subjects of HAP with PCT>0.25 μg/L. As shown in Figure 3C, Spearman's correlation test showed that a good correlation was found between the ratio of Tfh/Th with PCT level (r=0.370, *P*= 0.02).

3.6 The combination of Tfh/Th and PCT shows better value to predicting deteriorated HAP than PCT alone

We next hypothesized whether the ratio of Tfh/Th could predict the progression of HAP. PCT alone had an AUC of 0.703 (95% CI, 0.57-0.83), with the sensitivity of 88.89% and the specificity of 50%, respectively, when the critical value was 0.77. Tfh/Th alone had an AUC of 0.733 (95% CI, 0.61-0.86), with the sensitivity of 74.07% and the specificity of 70.59%, respectively, when the critical value was 0.15. As expected, the combination of Tfh/Th and PCT had an AUC of 0.807 (95% CI, 0.70-0.92), with the sensitivity of 74.07% and the specificity of 82.35% to predict the deterioration of HAP (Figure 4). These data indicated that the addition of Tfh/Th could increase the potential of PCT as a biomarker to predict the deterioration of HAP.

3.7 The ratio of Tfh/Th influenced the survival of HAP patients.

Lastly, we investigated the prognostic value of Tfh/Th in HAP. Given the critical value of Tfh/Th in predicting deteriorated HAP, we divided the individuals into two groups by the value of 0.15. By Kaplan-Meier survival analysis, the 28-day mortality of HAP patients with high (>0.15) and low (≤ 0.15) Tfh/Th ratios was assessed after the test day. As shown in Figure 5, HAP patients with high Tfh/Th ratio had a decreased rate of survival in 28 days, comparing to patients with low Tfh/Th ratio. These data suggest that the ratio of Tfh/Th could be used as a predictor of survival rate in HAP.

Table 1. Subject characteristics

Characteristics	n=62
Age(y)	18-85
≧65	25
<65	37
Gender	
male	38
female	24
Underlying diseases	19
Intracerebral hemorrhage	10
Cerebral embolism	6
Pelvic fracture	6
Renal failure	10
Pulmonary contusion	11
Brain contusion	
Clinic status	
Uncontrolled	34
Controlled	28
Pathogens	
Kp	21
Non-Kp	41
HAP with BSI	10
Yes	52
No	

4. Discussion

HAP is a major health burden worldwide. It is reported that immune dysregulation is closely associated with HAP in ICU patients [10]. Patients in ICU usually have received various invasive treatments, and these treatments could lead to excessive inflammatory response with altered cell-mediated immunity. The dysregulated immune response can subsequently increase susceptibility to infection and causing HAP [10]. In particular, the immune response regulation in HAP is dependent on complex interactions between alveolar macrophages, polymorphonuclear leukocytes, immune cells and local production of both pro- and anti-inflammatory cytokines as well as vascular adhesion molecules [11]. Tfh cells play an indispensable role in the formation and maintenance of germinal centers for ongoing immune responses. Current research on the relationship of HAP with CXCR5+CD4+T cells is relatively scarce. In our current study, we have identified a Tfh cell subset (CD4+FoxP3-CXCR5+ cells) that is increased in progressive pneumonia and K.p-positive pneumonia. Further analysis has also shown that Tfh/Th can be a better diagnostic biomarker when assessing the severity of HAP than PCT. The findings of our study reveal the involvement of CD4+ Tfh cells in HAP, and further provide a useful predictive value for the prognosis of HAP.

K.p is the leading cause of serious respiratory tract infections and the mortality rate of K.p-induced pneumonia can exceed 50%[12]. In addition, the increasing multidrug resistant K.p strains pose a major medical problem worldwide [13, 14]. In this study we also found the patients of HAP were mainly infected with K.p(n=21).K.p has two distinctive subgroups, the classic K.p and the hypervirulent K.p. The classic K.p is notoriously known to gain antimicrobial resistance, while the hypervirulent K.p has an even higher antibiotic-resistant rate and causes more severe diseases[15]. Therefore, the identification of K.p-infection in pneumonia diagnosis is important for the treatment of the disease. In the current study, we have identified that Tfh/Th can be used to differentiate K.p and non-K.p infected pneumonia. It would be valuable to investigate if this biomarker can be used alone or in combination with other diagnostic markers to facilitate the identification of K.p-infected pneumonia. In addition, CD4+FoxP3-CXCR5+ cells are a subgroup of Tfh cells which are essential for the development and maintain B cell immune responses [16]. Therefore, it would be warranted to study if Tfh cells are involved in anti-K.p immune response and the findings will provide valuable information for the development of better treatment strategies against K.p infection.

In ICU, BSI is another common complication in addition to HAP. Risk factors associated with BSI are multi-faced, which include not only the patient’s underlying conditions, but also therapeutic, microbial and environmental factors [17]. For HAP patients with BSIs, the immune system is further altered, and the balance between protective immunity and harmful hyper-inflammation is hard to be achieved [18]. The close relation of HAP and BSI to the dysregulated immune system explains that the change of Tfh/Th immune cell ratio could serve as a biomarker when HAP is complicated with BSI.

There is currently not enough research on bacterial pneumonia-related markers that can represent a definite diagnosis of bacterial pneumonia[19]. In addition, discovery of biomarkers that can differentiate

viral and bacterial pneumonia is also of great importance, in order to avoid unnecessary use of antibiotics[20]. In an inpatient setting, CRP, WBC and PCT are usually part of the diagnostic workup[8, 21]. However, the changes of these parameters are not always specific to predict causative pathogen. At preclinical and clinical levels, several new biomarkers like MxA1, HMGB1 and CXCR5+CD8+ T cells have shown more promising results. But rise or drop of a single marker still is not accurate enough to predict viral/bacterial pneumonia [6, 22]. In current study, we have identified another CD4 co-receptor expressing Tfh cell subclass as a promising biomarker for assessing the deterioration of HAP. Based on the ROC analysis, the combination of Tfh/Th and PCT is a better marker than the classical PCT alone. Combination of two or more markers gives better predictive accuracy. In addition, our study has also shown that the critical value of Tfh/Th can be a good prognostic marker for HAP survival.

Our current study and previous study have shown that CD4+ Tfh cells are associated with HAP however, the underlying mechanisms remain to be further investigated. In fact, the involvement of Tfh cells in the pathogenesis of bacterial pneumonia is in general under investigated. Tfh cells are usually CD4+, but populations of CD8+ Tfh cells have also been discovered [23, 24]. These cells have a number of cell surface markers including CXCR5, PD1 and ICOS, and they can be found in the B cell zone of secondary lymphoid organs and also in circulation [25, 26]. Tfh cells are essential for the development and maintain B cell immune responses, by promoting B cell proliferation and maturation with co-stimulating signals [4]. The relationship between Tfh cells and germline center B cells is positively related and the failure of germline center formation and defects of antibody production are observed in the absence of Tfh cells [27]. Given the importance of Tfh cells in the initiation and maintenance of B cell immune responses, it is rational to speculate that the elevation of CD4+ Tfh cells in bacterial pneumonia is accompanied with host immune responses after pathogen infection in the low respiratory sites. Although beyond the scope of our current study, it is interesting to investigate the importance of Tfh cell function in bacterial pneumonia.

Abbreviations

ATS/ERS: American Thoracic Society/European Respiratory Society;

AUC: Area under the curve;

BSIs: Bloodstream Infections;

COPD: Chronic obstructive pulmonary disease;

CRP: C-reactive protein;

CXCR5: C-X-C chemokine receptor type 5;

HAP: Hospital-acquired pneumonia;

PCT: Procalcitonin;

ROC: Receiver operating characteristic curve;

SD: Standard deviation;

Tfh cell: Follicular helper T cell;

Th cell: Helper T cell;

VAP: Ventilator-associated pneumonia;

WBC: White blood cell count.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the First People's Hospital of Kunshan Affiliated to Jiangsu University (Approval No.2019-112-2).

Consent for publication

Written informed consent was obtained from the participants or legal representatives.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the Project of Suzhou City (SYS02020040) and the Kunshan Science and Technology Planning Project (KS18060) and the Project of Suzhou Science and Technology (SKY2021034).

Authors' contributions

CC participated in the conception, the design and coordination of the study, collected data, and drafted the manuscript. PY and TT conceived of the study, and participated in its design and coordination and helped to draft the manuscript. YNW participated in collected data. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank all project study volunteers, clinical team members. We thank the engineer of flow cytometry cytometer, Qin-yue Wang, and Sha-sha Li for technical assistance.

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Figures

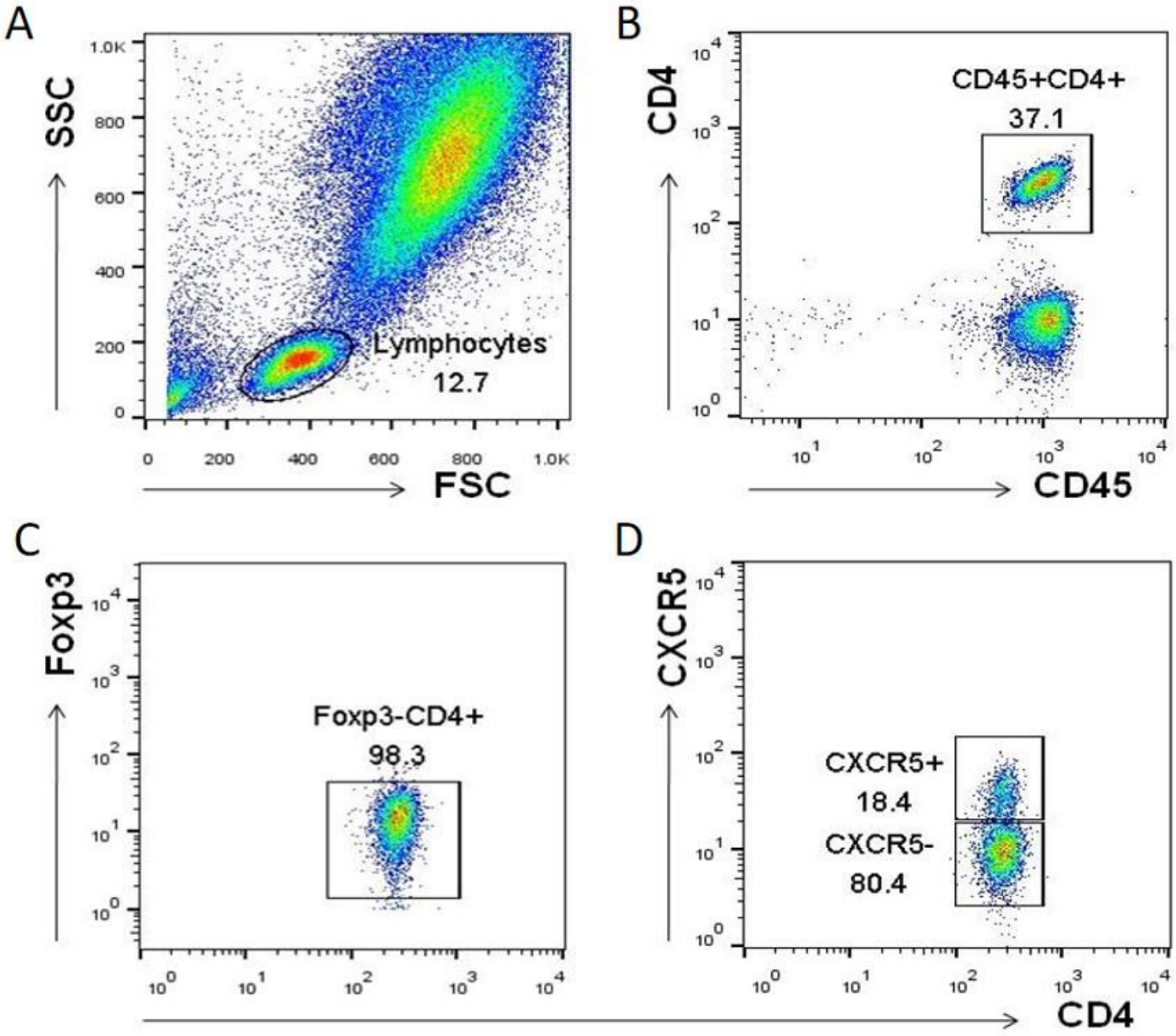


Figure 1

Analysis of Tfh and Th cells in all individuals by FCM. A, lymphocyte population was gated according to cell size and complexity, B CD45+CD4+T cell was gated from lymphocyte, C, Foxp3-CD4+T cell was gated from CD45+CD4+T, D, CXCR5+/-CD4+ T cell was gated from Foxp3-CD4+T cell. Representative dot plots are shown.

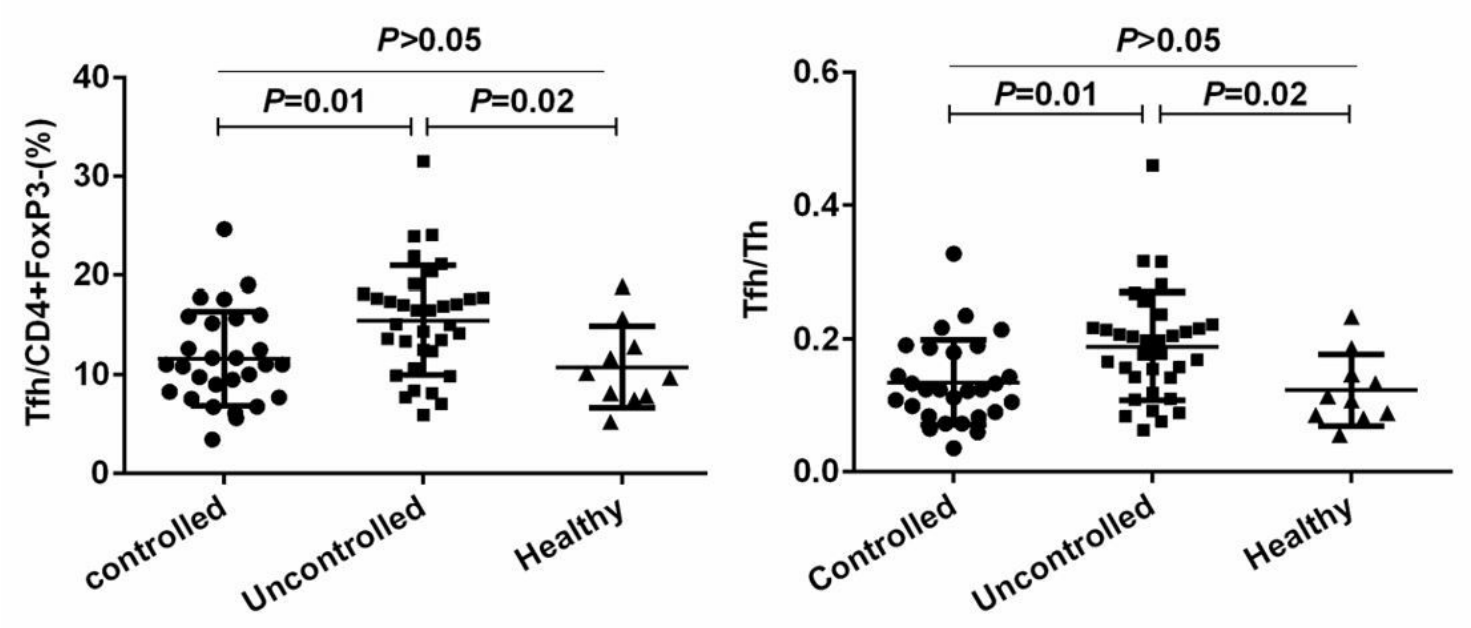


Figure 2

Circulating Tfh and Tfh/Th are increased in patients with uncontrolled HAP compared to controlled HAP and healthy group. Results are presented as the mean with SD levels were compared by Student's s t-test.

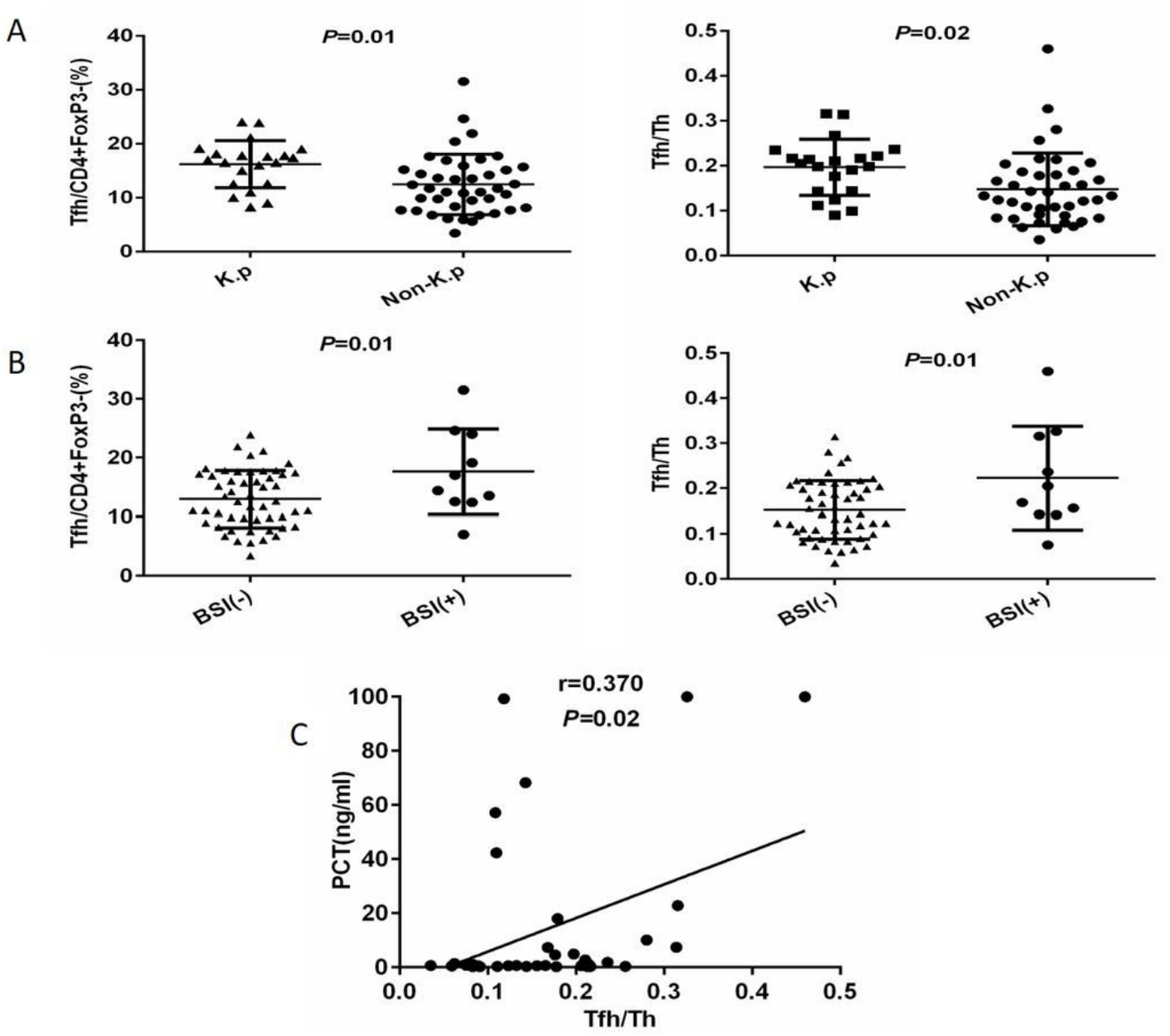
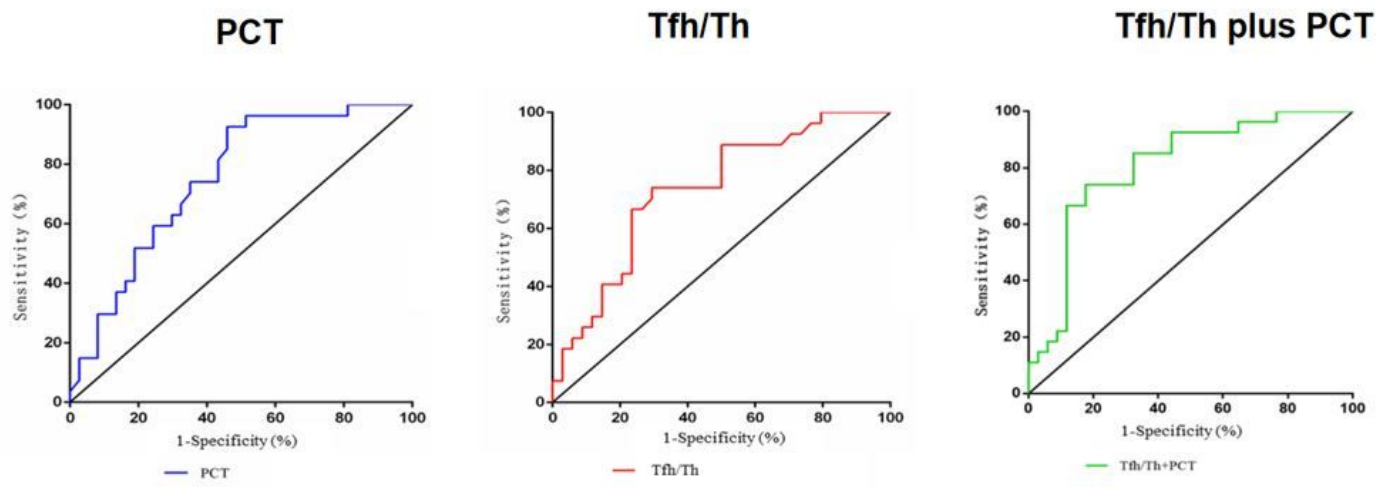


Figure 3

Comparison of Tfh and Tfh/Th in HAP patients with K.p and non-K.p infection (A), with BSI and without BSI (B). The correlation between PCT and the ratio of Tfh/Th in HAP was showed in C.



Marker	AUC	95%CI	Sensitivity（%）	Specificity（%）	Cut-off
PCT (ng/ml)	0.703	0.573-0.833	88.89	50	0.77
Tfh/Th	0.733	0.606-0.859	74.07	70.59	0.15
Tfh/Th plus PCT	0.807	0.695-0.919	74.07	82.35	0.38/0.15

Figure 4

The combination of Tfh/Th and PCT is a better predicting biomarker for the deterioration of HAP. The ROC curves for PCT (A) and Tfh/Th (B)and Tfh/Th plus PCT (C) were shown. The 95% CI, sensitivity, specificity and cut-off for Tfh/Th, PCT and Tfh/Th plus PCT were shown in D.

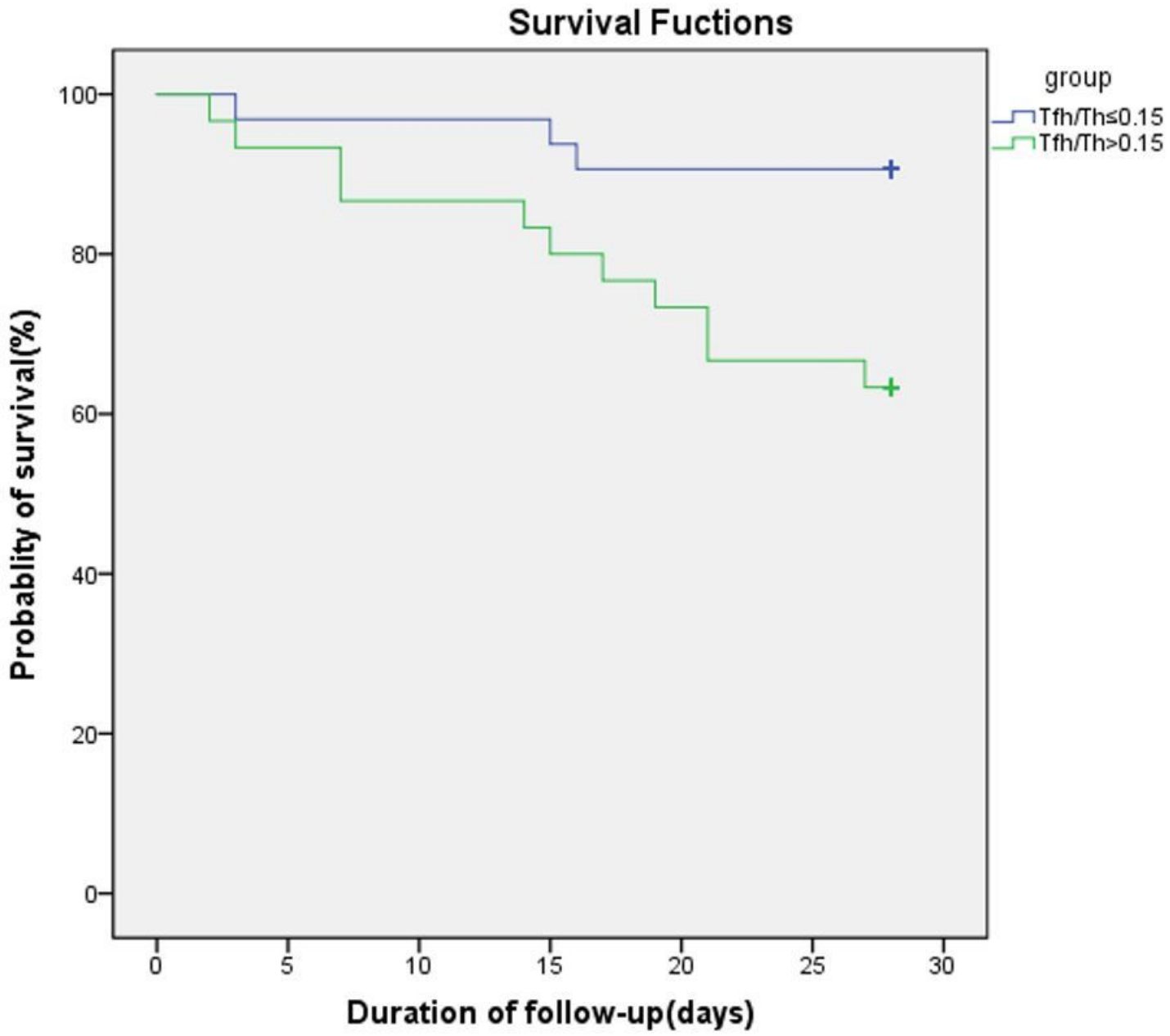


Figure 5

Survival analysis. Kaplan-Meier curves were estimated for the 28-day mortality of HAP patients with high and low Tfh/Th ratios. Statistical difference was analyzed by the long rank test.