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Prospective evaluation of lymphocyte subtyping for the diagnosis of invasive candidiasis in non-neutropenic critically ill patients

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ABSTRACT

Objectives: This study aimed to investigate the distinguishing ability of lymphocyte subtyping for Invasive candidiasis (IC) diagnosis and prognosis in non-neutropenic critically ill patients.
Methods: We assessed the quantitative changes in key parameters of lymphocyte subtyping at the onset of clinical signs of infection in non-neutropenic critically ill patients and their potential influence on diagnosis and outcome of IC. The primary outcome was 28-day mortality.
Results: Among the 182 consecutive critically ill patients, 22 (12.1%) were in the IC group. The $CD28^+CD8^+$ T-cell counts (AUC 0.863, 95%CI 0.804–0.909, $P<0.001$) had greater diagnostic value for IC than other parameters had. Adding $CD28^+CD8^+$ T to Candida score significantly improved the predictive value of Candida score ($P=0.039$). Multivariate logistic regression analysis identified $CD28^+CD8^+$ T-cell counts ≤ 78 cells/mm^3 (OR 24.544, 95%CI 6.461–93.236, $P<0.001$) as an independent predictor for IC diagnosis. $CD28^+CD8^+$ T-cell counts could also predict 28-day mortality. Kaplan–Meier survival analysis provided evidence that $CD28^+CD8^+$ T-cell count <144 cells/mm^3 (log-rank test; $P=0.03$) were associated with lower survival probabilities.
Conclusions: $CD28^+CD8^+$ T-cell counts play an important role in early diagnosis of IC. Low counts are associated with early mortality in non-neutropenic critically ill patients. These results suggest the potential usefulness of measuring $CD28^+CD8^+$ T-cell lymphocyte levels in the early recognition and diagnosis of IC.
Trial registration: ChiCTR-ROC-17010750. Registered 28 February 2017.

Introduction

Invasive candidiasis (IC) is a frequently occurring life-threatening complication in critically ill patients (Bassetti et al., 2017). Yeast of the genus *Candida* are the predominant pathogens in the intensive care setting, causing a broad spectrum of presentations, including catheter-related candidemia, disseminated candidiasis, intra-abdominal candidiasis, etc. The incidence of candidemia is age-related with higher frequencies at both ends of the spectrum (Bassetti et al., 2017).

IC in critically ill patients is associated with considerable morbidity and mortality. The main challenge is making a timely diagnosis of IC since delayed initiation of appropriate antifungal therapy has been shown to increase morbidity and mortality, especially in critically ill patients (Bassetti et al., 2014). However, conventional culture-based microbiological tests are suboptimal

Abbreviations: IC, invasive candidiasis; ICU, intensive care unit; AUC, area under the curve; BDG, 1,3-β-D-glucan; GM, galactomannan; PUMCH, Peking Union Medical College Hospital; APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, Sequential Organ Failure Assessment; NK, natural killer; ROC, Receiver operating characteristic curves; OR, odds ratio; CI, confidence interval; GNB, gram-negative bacteria.

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for IC diagnosis. Microbiological cultures are insensitive and take several days to obtain *Candida* species and antifungal susceptibilities (Clancy and Nguyen, 2013). Therefore, advanced diagnostic tools have been investigated, such as fungal antigen detection [1,3-β-D-glucan (BDG), *Candida albicans* germ tube antibody (CAGTA)], fugal DNA detection by PCR, and scoring systems. For example, Candida score (CS) is commonly used in ICU populations because of its convenience and efficiency (León et al., 2009). However, until now, no single factor mentioned above has allowed adequate and timely IC prediction in critically ill patients in clinical settings (Posteraro et al., 2011; Boch et al., 2016).

Control of fungal invasion relies on elements of both innate and adaptive immunity. Many large-scale prophylaxis trials prove the fact that mortality from IC remains elevated despite the use of antimicrobial agents that are highly active against fungal pathogens, implying that defective host immunity may contribute to the IC development and high mortality (Wenzel and Gennings, 2005). It is well known that critically ill patients usually have immune system dysfunction. Recent studies have shown evidence for host immune deficit predisposing to IC (Castellano-Gonzalez et al., 2017). Many investigations have found that some immunological parameters could provide information for determination of risk level and probable pathogens, including one study of our research group (Cui et al., 2013; Spec et al., 2016; Li et al., 2017). Whether these parameters could predict IC remains under discussion.

Thus, the objective of this prospective study was to evaluate the contribution of key immunological parameters such as lymphocyte subtyping for IC diagnosis and prognosis of critically ill patients.

Materials and methods

Patients and study design

A prospective study was performed at the Peking Union Medical College Hospital (PUMCH), covering the period from March 2017 to May 2018. PUMCH is a university hospital in China with >2,000 beds, including >40 ICU beds. This study was approved by the local institutional review board of PUMCH (No. of Ethics approval: JS-1170). Informed consent was obtained from all the involved patients, or more commonly next of kin. The study was registered at chictr.org.cn (identifier ChiCTR-ROC-17010750).

Inclusion criteria were as follows: (1) patients aged ≥18 years; (2) patients who required intensive care; (3) patients who were expected to stay for >48 h in ICU; and (4) patients with clinical signs of infection as diagnosed by the attending physician met at least two of the following clinical criteria: (i) body temperature ≥38 °C or <36 °C; (ii) respiratory rate ≥30 breaths/min; (iii) pulse rate ≥120 beats/min; and (iv) abnormal total peripheral white blood cell count ≥10,000/mm^3 or <4000/mm^3, or immature neutrophils >15% (Cui et al., 2017). We excluded patients who were pregnant or lactating, patients with neutropenia (absolute neutrophil count <500/mm^3) at baseline, patients with fungal infections other than those caused by *Candida* species, or those whose life expectancy was <48 h. Diagnosis of IC was defined with clinical signs of infection and at least one of the following criteria: (i) histopathological, cytopathological or direct microscopic confirmation of yeast cells in a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes); (ii) at least one peripheral blood culture positive for *Candida*; (iii) positive *Candida* culture from a sample obtained by sterile technique from a normally sterile site (e.g. cerebrospinal, pleural, peritoneal or peritoneal abscess fluid) (De Pauw et al., 2008; Guo et al., 2013). Timing to central venous catheter (CVC) removal and to antifungal administration was the interval between onset of infection and implementation of these measures. Adequate antifungal treatment was the use of the correct dose of antifungal agent for a susceptible *Candida* isolate (Puig-Asensio et al., 2014).

Clinical and laboratory evaluation

A comprehensive clinical assessment was done in all patients at the onset of clinical signs of infection. The assessment included age, sex and underlying diseases in association with IC development in critically ill patients (Bassetti et al., 2017). We also calculated the Acute Physiology and Chronic Health Evaluation (APACHE) II (Knaus et al., 1985) and Sequential Organ Failure Assessment (SOFA) (Vincent et al., 1996) scores at the onset of infection. Life-sustaining treatments included mechanical ventilation, vasopressors and renal replacement based on clinical assessment and recent recommendations (Rhodes et al., 2017). Treatment-related factors were also analyzed, including the percentage of indwelling catheters and drug therapy (such as antibiotics, corticosteroids and intravenous immunoglobulin). Follow-up included 28-day all-cause mortality rates from the day of enrollment.

All the specimens were performed immediately at the onset of clinical signs of infection, and were sent to the PUMCH clinical microbiology laboratory, which is also called Beijing Key Laboratory for Mechanisms Research and Precision Diagnosis of Invasive Fungal Diseases, as soon as possible. Antifungal susceptibility testing was performed using ATB™ FUNGUS 3 test kits (bioMérieux®, France) following the manufacturer's instructions. Empirical antibacterial and antifungal treatment was promptly initiated according to international recommendations (Freifeld et al., 2011; Pappas et al., 2016). The relevant CT scans, specimen cultures and other results within the week before enrollment were taken into consideration for IC diagnosis. CS was calculated utilizing the scoring system proposed by Leon et al, when results of the patient's cultures were available, with total parenteral nutrition × 1, plus surgery × 1, plus multifocal *Candida* colonization × 1, plus severe sepsis × 2 (León et al., 2009). Severe sepsis was defined according to the Sepsis-3 statements (Seymour et al., 2016). Two intensivists evaluated all *Candida*-positive cultures and other related clinical data to classify patients involved as IC group or no IC group according to the diagnostic criteria mentioned above. If they disagreed, a third intensivist resolved the disagreement.

Peripheral blood samples were simultaneously collected at the onset of clinical signs of infection for measurement of BDG, immunological parameters and lymphocyte subtyping by PUMCH laboratories. Peripheral blood mononuclear cells were separated and stained with combinations of different fluorescent monoclonal antibodies, followed by flow cytometric analysis (three-color EPICS-XL flow cytometer; Beckman Coulter, Brea, CA, USA) to detect T cells ($CD3^+$), $CD4^+$ T-cell subgroups ($CD4^+CD3^+$ and $CD28^+CD4^+$), $CD8^+$ T-cell subgroups ($CD8^+CD3^+$ and $CD28^+CD8^+$), B cells ($CD19^+$), and natural killer (NK) cells ($CD3^-CD16^+CD56^+$). Rate nephelometry (Array 360; Beckman Coulter) was used to measure serum levels of IgA, IgG and IgM, and complement factor (C) 3 and C4. An independent clinical research organization conducted monitoring according to good clinical practice and standard operating procedures in compliance with Chinese government regulations.

Statistical analysis

Normally distributed data were expressed as means and standard deviations, and were compared using Student's *t*-test. Non-normally distributed data were expressed as the median and interquartile range and were analyzed with the Mann–Whitney *U*

test. Categorical variables were recorded as proportions and compared using the χ^2 or Fisher's exact test. Receiver operating characteristic (ROC) curves were constructed for immune parameters to distinguish the diagnostic value and prognosis of IC. For comparison of the areas under the curve (AUC), $Z = (A_1 - A_2)/\sqrt{SE_1{}^2 + SE_2{}^2}$ was used, the test values being $Z_{0.05} = 1.96$ and $Z_{0.01} = 2.58$. Univariate and multivariate logistic regression analyses were performed to determine independent risk factors for IC diagnosis and prognosis, considering all variables with $P < 0.05$ on univariate analysis. The results were expressed as P and odds ratio (OR) with 95% confidence interval (CI). Kaplan–Meier survival analysis was used to construct survival curves, and comparisons of survival distributions were based on the log-rank test. The significance level was set at a two-sided P value of 0.05. Statistical analysis was performed using SPSS version 22.0 (SPSS, Chicago, IL, USA).

Results

Patients' characteristics

As reported in Figure 1, a total of 182 consecutive patients were involved in this prospective study. Table 1 shows the main characteristics of the study population. On the basis of the diagnostic criteria, there were 22 patients in the IC group. No difference was found between the IC and no-IC groups in the proportion of underlying diseases which have been reported in association with IC development in critically ill patients. In addition, there was no difference in APACHE II or SOFA score between the two groups.

In the IC group, four patients with candidemia, twelve with intra-abdominal infection and six with intra-pleural infection were infected with *Candida* spp, including fourteen with *Candida albicans*, four with *Candida glabrata* and two each with *Candida parapsilosis* or *Candida tropicalis*. Among the 22 patients with IC, 5 (22.7%) died during the follow-up period, with a mean of 10.2 days from enrollment to death. About antifungal susceptibility results, for fluconazole, 90.9% (20/22) of *Candida* isolates were susceptible, with one non-susceptible isolates of *Candida albicans* and one non-susceptible isolate of *Candida glabrata*. There was no resistance to echinocandins.

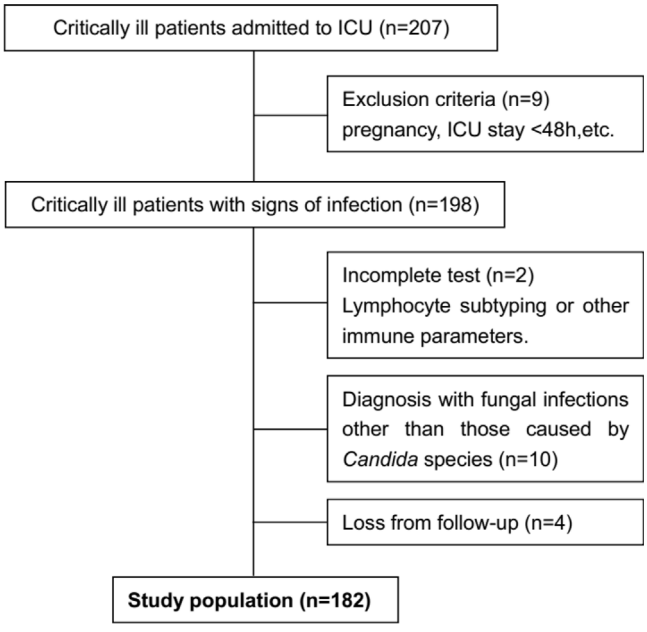

Figure 1. Flow chart describing the study population.

Table 2 shows the clinical characteristics of all the patients at the onset of clinical signs of infection. There was no significant difference in life-sustaining treatments and indwelling catheters between the groups. More patients in the IC group received antifungal drugs and corticosteroids. Procalcitonin, BDG and C-reactive protein level did not differ between the groups. There was no difference in coexisting pathogens between the two groups. The IC group had higher level of CS (4.41 ± 0.80 vs. 3.53 ± 1.12, $P < 0.001$). The 28-day mortality was significantly higher in the IC group (33.3 vs. 7.0%, $P = 0.001$).

Comparison of immune parameters by IC diagnosis

$CD8^+$ [115.0 (119.5) vs. 235.5 (233.8), $P = 0.009$] and $CD28^+CD8^+$ [47.0 (53.0) vs. 121.5 (133.8), $P < 0.001$) T-cell counts were significantly lower in the IC than no IC group. There were no significant differences in other immune parameters between the two groups (Table 3). To evaluate the ability of immune parameters for IC diagnosis, ROC analysis was performed with the immunological parameters that differed significantly between the IC and no IC groups (Table 4). The $CD28^+CD8^+$ T-cell counts (AUC 0.863, 95%CI 0.804–0.909, $P < 0.001$) had greater diagnostic value for IC than other immune parameters had. The cut-off value of $CD28^+CD8^+$ T-cell counts for IC diagnosis was 78 cells/mm^3, with sensitivity of 86.4% and specificity of 75.0%. The $CD8^+$ T-cell counts and CS also had diagnostic values for IC diagnosis. We also applied ROC analysis with BDG, finding that AUC of BDG (0.579, 95%CI 0.500–0.655, $P = 0.243$) was less than that of immune parameters. The ROC curves for $CD28^+CD8^+$ T-cell counts and CS were shown in Figure 2. The AUC for $CD28^+CD8^+$ T in predicting IC were larger than that of CS (0.863 vs. 0.760, $P = 0.028$). Adding $CD28^+CD8^+$ T to CS significantly improved the predictive value of CS ($P = 0.039$). However, there was no significant difference in the AUCs of $CD28^+CD8^+$ T and adding $CD28^+CD8^+$ T to CS ($P = 0.826$).

Multivariate logistic regression analysis was conducted for $CD28^+CD8^+$ T-cell counts ≤ 78 cells/mm^3 and the variable that demonstrated $P < 0.05$ in univariate analysis (CS). Multivariate logistic regression analysis (Table 5) identified two independent risk factors for IC diagnosis in critically ill patients: $CD28^+CD8^+$ T-cell counts ≤ 78 cells/mm^3 (OR 24.544, 95%CI 6.461–93.236, $P < 0.001$) and CS (OR 3.245, 95%CI 1.635-6.439, $P = 0.001$). As $CD28^+CD8^+$ T-cell counts depended on the $CD8^+$ T-cell counts, we applied multivariate logistic regression analysis separately. Multivariate logistic regression also identified $CD8^+$ T-cell counts ≤ 143 cells/mm^3 (OR 8.909, 95%CI 3.112-25.505, $P < 0.001$) as an independent risk factor.

Comparison of immune parameters in survivors and non-survivors

We divided all the patients into survivors and non-survivors according to 28-day mortality. Compared with the non-survivors, NK cells ($P = 0.024$), $CD8^+$ T-cell counts ($P = 0.010$) and $CD28^+CD8^+$ T-cell counts ($P = 0.016$) were significantly higher in survivors. There were no differences in other immune parameters studied between survivors and non-survivors (Table 6).

To evaluate the distinguishing ability of prognosis, ROC analysis was also applied to the immune parameters that were significantly different between survivors and non-survivors according to 28-day mortality. NK-cell counts, $CD8^+$ T-cell counts and $CD28^+CD8^+$ T-cell counts could predict 28-day mortality, with AUC values shown in Table 7. We constructed Kaplan–Meier survival curves, showing that NK-cell count <80 cells/mm^3 (log-rank test; $P < 0.001$), $CD8^+$ T-cell count <236 cells/mm^3 (log-rank test; $P = 0.007$) and $CD28^+CD8^+$ T-cell count <144 cells/mm^3 (log-rank test; $P = 0.03$)

Table 1
Characteristics of the study population.

Variables	All patients (n = 182)	No IC (n = 160, 87.9%)	IC (n = 22, 12.1%)	*P* value*
Mean age (y)	61.6 ± 16.0	61.5 ± 15.9	62.8 ± 17.1	0.725
Gender, male	103 (56.6)	95 (59.4)	8 (36.4)	0.065
Underlying disease, n(%)				
COPD	7 (3.8)	6 (3.8)	1 (4.5)	0.856
Diabetic mellitus	44 (24.2)	38 (23.8)	6 (27.3)	0.717
Chronic renal failure	12 (6.6)	11 (6.9)	1 (4.5)	0.680
Hepatic failure	3 (1.6)	3 (1.9)	0 (0.0)	0.517
Solid tumor	41 (22.5)	35 (21.9)	6 (27.3)	0.570
Immune system disease	14 (7.7)	12 (7.5)	2 (9.1)	0.793
Hematological disease	3 (1.6)	3 (1.9)	0 (0.0)	0.517
APACHE II score	16.47 ± 6.33	16.38 ± 6.26	17.25 ± 6.98	0.562
SOFA score	8.84 ± 4.20	8.77 ± 4.09	9.40 ± 5.08	0.530

APACHE II, Acute Physiology and Chronic Health Evaluation II; COPD, chronic obstructive pulmonary disease; SOFA, Sequential Organ Failure Assessment.
* *P* value for the comparison between no IC and IC groups.

Table 2
Clinical characteristics of critically ill patients.

Variables	All patients (n = 182)	No IC (n = 160, 87.9%)	IC (n = 22, 12.1%)	*P* value*
Infection marker at the onset of signs of infection				
PCT level (ng/ml)	0.94 (3.29)	0.92 (2.77)	1.53 (14.67)	0.052
BDG level (pg/ml)	53.2 (67.6)	37.6 (69.3)	58.3 (70.6)	0.729
CRP level (mg/L)	66.18 (118.99)	73.58 (113.16)	51.52 (195.92)	0.513
Coexisting pathogens, n(%)				
Bacteria	140 (76.9)	124 (77.5)	16 (72.7)	0.618
CMV	17 (9.3)	12 (7.5)	5 (22.7)	0.067
PCP	4 (2.2)	3 (1.9)	1 (4.5)	0.423
Life-sustaining treatments, n(%)				
Need for mechanical ventilation	161 (88.5)	141 (88.1)	20 (90.9)	0.702
Need for vasopressor	133 (73.1)	115 (71.9)	18 (81.8)	0.324
Need for RRT	37 (20.3)	31 (19.4)	6 (27.3)	0.388
Indwelling catheter, n(%)				
Urinary catheter	170 (93.4)	149 (93.1)	21 (95.5)	0.680
CVC	154 (84.6)	136 (85.0)	18 (81.8)	0.698
CVC removal	58 (31.9)	44 (27.5)	14 (63.6)	0.001
Drug therapy, n(%)				
Adequate antifungal drugs	45 (24.7)	28 (17.5)	17 (77.3)	< 0.001
Corticosteroids	31 (17.0)	23 (14.4)	8 (36.4)	0.010
IVIG	4 (2.2)	2 (1.3)	2 (9.1)	0.072
Antibiotics for GNB	140 (76.9)	121 (75.6)	19 (86.4)	0.262
Antibiotics for GPB	114 (62.6)	99 (61.9)	15 (68.2)	0.566
TMP-SMX	8 (4.4)	7 (4.4)	1 (4.5)	0.971
Total parenteral nutrition, n(%)	158 (86.8)	138 (86.3)	20 (90.6)	0.530
Surgery, n(%)	118 (64.8)	101 (63.6)	17 (77.3)	0.177
Candida score	3.64 ± 1.12	3.53 ± 1.12	4.41 ± 0.80	< 0.001
ICU duration (days)	6 (10)	6 (10)	8 (9)	0.958
Hospital duration (days)	16 (18)	17 (19)	11 (14)	0.087
28-day mortality, n(%)	19 (10.4)	14 (8.8)	5 (22.7)	0.044

BDG, (1,3)-β-D-glucan; CMV, cytomegalovirus; CRP, C-reactive protein; CVC, central venous catheter; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; IVIG, intravenous immunoglobulin; PCP, Pneumocystis carinii pneumonia; PCT, procalcitonin; RRT, renal replacement therapy; TMP-SMX, trimethoprim/sulfamethoxazole. CVC removal means CVC removal within 48 h.
* *P* value for the comparison between no IC and IC groups.

were associated with lower survival probabilities in our study population (Figure 3). As $CD28^+CD8^+$ T-cell counts depended on the $CD8^+$ T-cell counts, we applied multivariate logistic regression analysis separately. Adequate antifungal treatment and CVC removal remained in the final multivariate analysis, given the belief that these factors might be associated with outcome shown in CANDIPOP study (Puig-Asensio et al., 2014). Multivariate analysis identified two independent risk factors for 28-day mortality: $CD8^+$ T-cell counts (OR 0.995, 95%CI 0.990–0.998, $P = 0.036$), $CD28^+CD8^+$ T-cell counts (OR 0.993, 95%CI 0.985–0.998, $P = 0.047$). NK-cell counts (OR 0.991, 95%CI 0.981–1.001, $P = 0.087$) was not an independent risk factor in the multivariate analysis.

Discussion

Over the last few decades, IC has been known as an important complication of critical illness associated with considerable morbidity, mortality and increased healthcare costs. Unfortunately, its diagnosis is problematic, given nonspecific clinical features, relatively slow turnaround times for microbiological culture results, and practical difficulties in sampling potential deep tissue foci. New diagnostic tools such as BDG detection are not yet universally available or standardized. Although CS is popular in predicting IC (León et al., 2009; Posteraro et al., 2011; Bruyere et al., 2014), it requires the results of fungal cultures which need at least

Table 3
Analysis of immune parameters in critically ill patients according to IC diagnosis.

Parameters	All patients (n = 182)	No IC (n = 160, 87.9%)	IC (n = 22, 12.1%)	*P* value*
WBC (cells/mm^3)	12220 (9040)	12000 (8842.5)	14520 (9955)	0.580
LY (cells/mm^3)	993 (732.0)	1028 (716.3)	666 (528.5)	0.071
NK (cells/mm^3)	72.0 (93.5)	72.5 (95.3)	72.0 (105.5)	0.687
LB (cells/mm^3)	143.0 (163.5)	143.5 (170.0)	135.0 (115.5)	0.487
CD3+T (cells/mm^3)	724.0 (617.5)	758.0 (578.8)	428.0 (469.0)	0.072
CD4+T (cells/mm^3)	455.0 (423.5)	494.5 (412.3)	241.0 (329.5)	0.160
CD28+CD4+T (cells/mm^3)	436.0 (432.0)	456.5 (419.8)	203.0 (294.0)	0.163
CD8+T(cells/mm^3)	208.0 (239.0)	235.5 (233.8)	115.0 (119.5)	0.009
CD28+CD8+T (cells/mm^3)	108.0 (107.0)	121.5 (133.8)	47.0 (53.0)	< 0.001
C3 (g/L)	0.826 (0.370)	0.844 (0.349)	0.703 (0.484)	0.088
C4 (g/L)	0.174 (0.083)	0.175 (0.374)	0.148 (0.091)	0.074
IgA (g/L)	2.03 (1.61)	2.04 (1.59)	1.98 (1.78)	0.712
IgG (g/L)	9.09 (5.05)	9.08 (5.04)	9.20 (7.43)	0.845
IgM (g/L)	0.71 (0.63)	0.76 (0.68)	0.54 (0.52)	0.311

C3, complement factor 3; C4, complement factor 4; Ig, immunoglobulin; LB, B lymphocyte; LY, lymphocyte; NK, natural killer; WBC, white blood cell. Continuous variables are expressed as means ± SD or medians (IQR).
* *P* value for the comparison between no IC and IC groups.

Table 4
Receiver operating characteristics curve analysis of immune parameters predicting IC.

Parameters	Cut-off value	AUC	95%CI	*P* value
$CD8^+$T(cells/mm^3)	143	0.779	0.711-0.837	<0.001
$CD28^+CD8^+$T (cells/mm^3)	78	0.863	0.804-0.909	<0.001
Candida Score	4	0.760	0.659-0.793	<0.001
BDG (pg/ml)	–	0.579	0.500-0.655	0.243

AUC, area under the curve; BDG, 1,3-β-D-glucan; CI, confidence interval.

two days. Besides, CS has a high negative predictive value but a low positive predictive value, which is not good for early diagnosis (León et al., 2009). As a result, diagnosis of IC is difficult in these critical care patients who do not have the host risk factors for IC as outlined in existing published guidelines. Given these factors and the increased mortality associated with delays in antifungal therapy initiation, a timely diagnostic approach is necessary, especially for critically ill patients. Lymphocyte subtyping is one of the suitable approaches, due to the ease of collecting peripheral blood samples and short duration of results available. The present study is believed to be the first prospective study to use lymphocyte subtyping assessing the quantitative changes in host immune status and the potential role of these immune parameters in the diagnosis and prognosis of IC in ICU patients. By studying a cohort of 182 critically ill patients, we found that $CD28^+CD8^+$ T-cell counts had larger AUC for IC diagnosis than that of CS, BDG and other immune parameters. CS also had diagnostic value in IC in our study population. However, $CD28^+CD8^+$ T-cell count is an earlier and better indicator of IC than CS, and adding $CD28^+CD8^+$ T-cell count can improve the diagnostic performance of CS. This result suggests that there is a potential in adding $CD28^+CD8^+$ T-cell count to CS for identifying patients with IC risk. Besides, lower $CD28^+CD8^+$ T-cell counts may be critical for prognosis prediction. This finding confirms the potential role of host immunity in the development of opportunistic infection, also provides evidence that evaluation of lymphocyte subtyping is important for early diagnosis and prognostic prediction in critically ill patients, which is helpful for clinical practice.

As is known to all, the ability of the host to survive IC requires a well-coordinated response by the innate and adaptive immune systems; both of which are often impaired in patients with IC (Lang et al., 2005; Boomer et al., 2011). Most patients with IC are immunosuppressed due to underlying disease, such as immune system diseases, solid tumor and hematological malignancy. However, there is increasing evidence that many ICU patients who are presumed to be immunocompetent also acquire IC. The

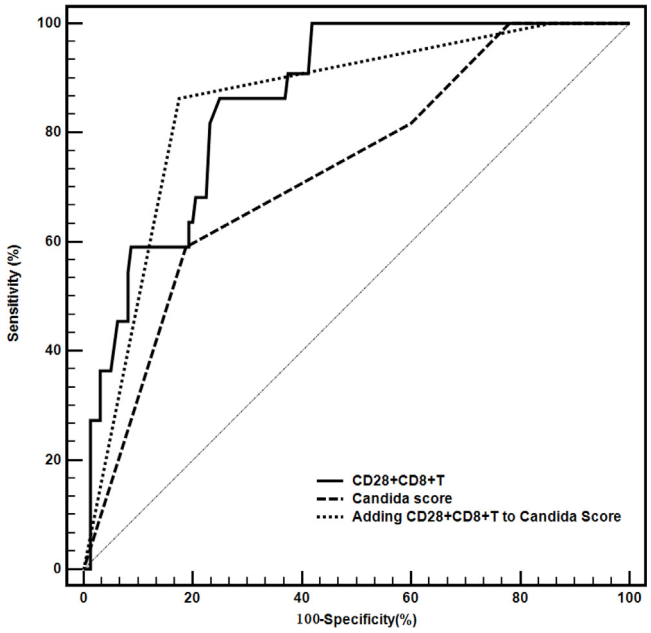

Figure 2. Receiver operating characteristic curves of $CD28^+CD8^+$T and Candida score in IC diagnosis. The AUC for $CD28^+CD8^+$ T in predicting IC were larger than that of Candida score (0.863 vs. 0.760, P = 0.028). Adding $CD28^+CD8^+$ T to Candida score significantly improved the predictive value of Candida score (P = 0.039). However, the AUC of $CD28^+CD8^+$ T was not significantly different from the AUC of adding $CD28^+CD8^+$ T to Candida score (P = 0.826).

immune response of critically ill patients may be greatly influenced by fungal infection (Lyn-Kew and Standiford, 2008). Therefore, in critically ill patients with IC, the interaction between pathogen and host could affect immune status, which should have prognostic value. We assessed levels of C3 and C4, IgA, IgG and IgM and lymphocyte subpopulations (T, B and NK cells), in critically ill patients at the onset of clinical signs of infection. We showed that

Table 5
Multivariate logistic regression analysis of independent factors predicting IC in critically ill patients.

Variables	OR	95%CI	*P* value
$CD28^+CD8^+$ T-cell counts ≤ 78 cells/mm^3	24.544	6.461-93.236	<0.001
Candida score	3.245	1.635-6.439	0.001

CI, confidence interval; OR, odds ratio.

Table 6
Analysis of immune parameters in critically ill patients according to 28-day mortality.

Parameters	All patients (n = 182)	Survivors (n = 160, 87.9%)	Non-survivors (n = 22, 12.1%)	*P* value*
WBC (cells/mm^3)	12220 (9040)	12060 (8730)	15205 (10652.5)	0.997
LY (cells/mm^3)	993 (732.0)	1000 (764.0)	895.5 (681.5)	0.136
NK (cells/mm^3)	72.0 (93.5)	78.0 (100.0)	41.0 (47.8)	0.024
LB (cells/mm^3)	143.0 (163.5)	142.0 (162.0)	164.5 (159.5)	0.785
CD3+T (cells/mm^3)	724.0 (617.5)	733.0 (620.0)	465.5 (495.5)	0.144
CD4+T (cells/mm^3)	455.0 (423.5)	484.0 (424.0)	315.0 (472.5)	0.316
CD28+CD4+T (cells/mm^3)	436.0 (432.0)	439.0 (423.0)	307.0 (469.3)	0.343
CD8+T(cells/mm^3)	208.0 (239.0)	224.0 (245.0)	157.0 (140.0)	0.010
CD28+CD8+T (cells/mm^3)	108.0 (107.0)	112.0 (127.0)	86.0 (76.0)	0.016
C3 (g/L)	0.826 (0.370)	0.841 (0.341)	0.665 (0.540)	0.559
C4 (g/L)	0.174 (0.083)	0.174 (0.084)	0.159 (0.105)	0.214
IgA (g/L)	2.03 (1.61)	2.03 (1.53)	2.31 (2.67)	0.837
IgG (g/L)	9.09 (5.05)	9.04 (4.79)	10.09 (10.39)	0.204
IgM (g/L)	0.71 (0.63)	0.70 (0.66)	0.85 (0.58)	0.742

C3, complement factor 3; C4, complement factor 4; Ig, immunoglobulin; LB, B lymphocyte; LY, lymphocyte; NK, natural killer; WBC, white blood cell. Continuous variables are expressed as means ± SD or medians (IQR).
* *P* value for the comparison between survivors and non-survivors.

Table 7
Receiver operating characteristics curve analysis of immune parameters predicting 28-day mortality in critically ill patients.

Parameters	Cut-off value	AUC	95%CI	*P* value
NK (cells/mm^3)	80	0.720	0.649–0.784	<0.001
CD8+T(cells/mm^3)	236	0.712	0.641–0.777	<0.001
CD28+CD8+T (cells/mm^3)	144	0.672	0.599–0.740	0.001

AUC, area under the curve; CI, confidence interval.

differences in the levels of these key immune parameters could reflect their clinical prognostic value.

IC represents an important paradigm in immunology, as they can result from either a lack of recognition by the immune system or over-activation of the inflammatory response (Romani, 2011). A variety of factors contribute to the impaired immunity in IC patients, including loss of immune effector cells, increased immunosuppressive cells (T regulatory cells and myeloid-derived suppressor cells), and T cell exhaustion (Boomer et al., 2011). T cell exhaustion is a condition that occurs following chronic antigen stimulation in which T cells become poorly functional with reduced cytokine production and decreased proliferative capacity, and are prone to undergo apoptotic cell death. An increase in T cell expression of the inhibitor receptor PD-1 and its ligand, PD-L1, are essential for mediating T cell exhaustion (Sharpe et al., 2007). Spec et al. (2016) found CD8$^+$ T cells from patients with candidemia had an increase in both the positive cells for PD-1 and the numbers of receptors for PD-L1 compared to controls, indicating that CD8$^+$ T cells act as a sensitive marker in T cell exhaustion related to IC. In this study, we found that lower CD8$^+$ and CD28$^+$CD8$^+$ T-cell counts at the onset of infection could predict IC and 28-day mortality. CD28$^+$CD8$^+$ T-cell count was the strongest lymphocyte subtype marker in early diagnosis of IC. This result is consistent with the outcome analysis depended on 28-day mortality and provides to support the important roles of CD8$^+$ T-cells as part of the host immune response to IC. Although CD4$^+$ T cells have a central role in coordination of the host defenses against fungi, the cytotoxic effect of CD8$^+$ T-cells seems ultimately to be essential for termination of the infection. This is coherent with present understanding of the pathophysiology of the host immune response, and explains the higher mortality rate in critically ill patients who manifest CD8$^+$ T cell deficiency. Previous studies have shown that CD4$^+$ T-cell responses are important for protection from IC. However, CD8$^+$ T cells have not been examined. Recent studies have focused on discovery of immune mechanisms against IC. These have revealed the protective roles of CD8$^+$ T cells during fungal infections, including release of antimicrobial peptides, production of the signature cytokine interferon γ and lysis of fungus-containing phagocytes (Huffnagle et al., 1991; Ma et al., 2002; Chaudhary et al., 2010). Even in the absence of CD4$^+$ T cells, CD8$^+$ T cells can protect mice from *Cryptococcus neoformans* infection. The fact that cytotoxic, class-I restricted, *A. fumigatus*-specific CD8$^+$ T-cell clones from human peripheral blood can expand also suggests that CD8$^+$ T cells contribute to cell-mediated defense (Lindell et al., 2005; Ramadan et al., 2005). In a recent study, a mouse model of *Candida* sepsis showed impaired CD8$^+$ T cell immunity, and enhancing CD8$^+$ T cell function by interleukin-7 improved outcome (Unsinger et al., 2012), which also demonstrated the vital role of CD8+ T cell in defending *Candida* species.

T-cell activation is carefully regulated by expression of positive and negative co-stimulatory molecules that prevent unbridled T-cell function. CD28 is the classic positive costimulatory receptor that, acting in conjunction with the T-cell receptor, implicates in a

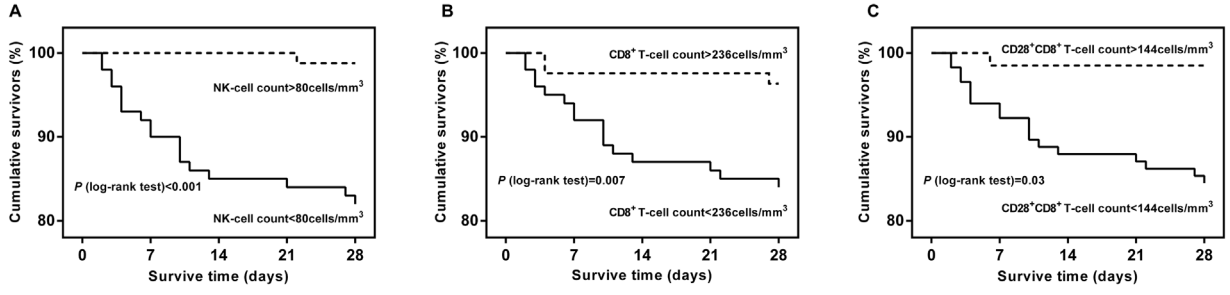

Figure 3. Kaplan-Meier analysis of survival probabilities in study population. (A) (B) (C) Survival measured according to NK-cell more (or less) than 80 cells/mm^3 and CD8$^+$ T-cell counts more (or less) than 236 cells/mm^3 and CD28$^+$CD8$^+$ T-cell counts more (or less) than 144 cells/mm^3 at the onset of clinical signs of infection. Survival time was censored on day 28.

wide array of T-cell responses, including T-cell proliferation, antigen-stimulated differentiation, activation and cytokine production (Sharpe and Abbas 2006; Ostrosky-Zeichner et al., 2014). Expression of CD28 on the surface of T-cells is greatly decreased in severely infected patients (Monserrat et al., 2009), and reduced CD28 expression is suggested to be an independent risk factor for higher mortality of patients with infection. Recent studies have shown that the requirement for CD28 co-stimulation differs between $CD4^+$ and $CD8^+$ T-cells and appears to depend on antigen presentation (Castellano-Gonzalez et al., 2017). All the above findings are consistent with our results. Our prospective study identified CD28 co-stimulation in $CD8^+$ T-cells, instead of $CD4^+$ T cells, as an independent risk factor for IC and early mortality in critically ill patients. This provides new evidence for the early diagnosis of IC in critically ill patients.

There were several limitations to this study. First, the existing EORTC/MSG definitions apply to immunocompromised patients but not necessarily to critically ill patients in ICU who, nonetheless, may develop IC. As a result, the IC diagnostic criteria used in our study were formulated based on EORTC/MSG definitions and other published studies (De Pauw et al., 2008; Guo et al., 2013). We are aware that the diagnostic criteria we utilized may have introduced bias and led to either over- or under-diagnosis of IC, but we believe this diagnostic criteria is suitable in the ICU setting. Second, owing to the influence of traditional cultures and the potentially devastating complications, autopsy and tissue biopsy were limited. This led to subsequent influence on the evaluation. However, this study can reflect the actual clinical settings in most developing countries to some extent. Third, this study included only a Chinese population, thus potentially reducing our ability to generalize the results. What's more, the mortality of our study population is lower than expected, probably due to the large number of intraabdominal infections. The number of episodes of IC is low and this fact does not allow us to make a subgroup analysis. A large-scale prospective study is needed to confirm our results.

Conclusion

Our study suggests that $CD28^+CD8^+$ T-cell counts play an important role in early diagnosis of IC. Lower $CD28^+CD8^+$ T-cell counts are associated with early mortality in non-neutropenic critically ill patients, and may be valuable for prognosis prediction. Our findings add evidence to the utility of lymphocyte subtyping in a diagnostic algorithm to better define IC in critically ill patients. New diagnostic criteria which incorporate these immune parameters may improve detection of patients with IC and contribute to antifungal stewardship programs in this patient cohort. $CD28^+CD8^+$ T-cell count is a potential diagnostic tool to identify high-risk patients and will work as a trigger for empirical therapy in future studies.

Ethics approval and consent to participate

This study was approved by the local institutional review board of PUMCH (No. of Ethics approval: JS-1170). Informed consent was obtained from all the patients involved.

Consent for publication

Not applicable.

Availability of data and materials

The data set supporting the results of this article is included within the article.

Declarations of interest

None.

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Authors' contributions

Jiahui Zhang designed the study and prepared the drafting of this article. Na Cui conceived the study and made final approval of this manuscript. Yun Long and Hao Wang made analysis of all data and helped revise this manuscript. Wen Han was in charge of acquisition of laboratory data and Yuanfei Li was in charge of acquisition of clinical data. Meng Xiao took charge of the acquisition of microbiological cultures' results.

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