

RESEARCH ARTICLE

Establishment of the reference intervals of whole blood neutrophil phagocytosis by flow cytometry

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Abstract

Objective: To investigate the reference intervals (RIs) of the whole blood neutrophil phagocytosis by flow cytometry (FCM) and to study the application value of neutrophil phagocytosis in infectious diseases.

Methods: Pathogens (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923) cultured for 18–24 h were labeled by fluorescence probe carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), and then incubated with whole blood at 37°C. The phagocytosis of pathogens by neutrophils was detected by flow cytometry, and a reference interval was established.

Results: In the healthy adults, the reference interval for the neutrophil phagocytosis to *Escherichia coli* was 46.91%–83.09% and to *Staphylococcus aureus* was 33.92%–69.48%. This method showed good reproducibility. Neutrophil phagocytosis was negatively correlated with the neutrophil count, neutrophil percentage, and neutrophil-to-lymphocyte ratio (NLR, $p < 0.05$).

Conclusion: We have successfully established the RIs of neutrophil phagocytosis in whole blood in healthy adults by flow cytometry (FCM), which might be of important clinical value in the diagnosis, treatment, and prognosis of infectious diseases.

KEYWORDS

flow cytometry, neutrophil, phagocytosis, reference intervals

1 | INTRODUCTION

About four million people die each year by infectious diseases over the world with a global mortality rate of 72.4 per 100,000.¹ Infection is one of the common causes of death in hospitals, especially with innate immunity impaired patients. Neutrophils are essential for the clearance of most infection-related pathogens. As one of the innate immune functions, phagocytosis of neutrophils plays a crucial role in the resistance to infection and the spread of pathogens. The realization of this function is to prevent the invasion and spread of pathogenic bacteria by engulfing and internalizing the pathogen into phagosomes and then killing them.² Defects in phagocytosis can

cause fatal infections.³ Phagocytic function analysis could be utilized as a vital tool to monitor the immune status of patients and provide good methodological parameters for the study of pathogenic mechanisms. However, the traditional detection methods used to evaluate phagocytosis are tedious, time-consuming, and subject to limitations of the examiner's abilities. Therefore, an objective, simple, rapid, and accurate method for the evaluation of phagocytosis is needed, which is made possible by the widespread use of flow cytometry.⁴

Neutrophils, as the dominant white blood cells, are considered as "specialized phagocytes". They work in cooperation with various humoral components of the body and are activated under the role

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of complement or inflammatory mediators, playing a particularly important role in the immune defense strategy.⁵ Some recent studies have shown that neutrophil phagocytosis is closely related to the development and outcome of some infectious diseases⁶ and tumors.⁷ Hence, the methods which can easily, rapidly, and accurately determine the function of neutrophil phagocytosis are still needed in clinical practice.

The traditional method of measuring neutrophil phagocytosis is to count 100 neutrophils with a microscope, record the number of neutrophils that have engulfed bacteria, and calculate the percentage to evaluate the phagocytic function. Flow cytometry is a high-throughput method that can measure tens of thousands of cells in a short time. With the widespread use of flow cytometry, scholars have made a series of new works and attempt to determine the phagocytosis of neutrophils, but they have not established a normal range that can evaluate the phagocytic function.⁸⁻¹⁰ In this study, we developed a simple method to quantify the neutrophil phagocytic function by using whole blood. Furthermore, we established the RIs of neutrophil phagocytosis in healthy adults. The established RIs have practical value based on the validation data in other healthy adults and infected patients.

2 | MATERIALS AND METHODS

2.1 | Study populations

One hundred and thirty one healthy individuals (71 males and 60 females) were enrolled at the Zhejiang Provincial People's Hospital, Hangzhou, China (Table 1). Exclusion criteria were as follows: diabetes, pregnancy, chronic nephropathy, hepatobiliary disease, allergic disease, autoimmune disease, hematological disease, fever, aged <18, and patients with disease in the acute phase. To validate this study, another 37 healthy adults (19 males, 18 females) who met the same inclusion criteria were recruited. An additional 38 infected patients (23 males, 15 females) were selected for this study who were positive for bacterial cultures in their body fluids and/or blood (Table 2). Exclusion criteria were radiation and/or chemotherapy, autoimmune disease, and hematological disease.

2.2 | Bacterial strains

Escherichia coli (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) provided by the Center of Clinical Laboratory Medicine at the Zhejiang Provincial People's Hospital were used. The strains were routinely grown in Columbia's blood agar plates for 18 h at 37°C and resuspended in sterilized saline to 10⁸/ml (OD = 1). Bacteria were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) in sterile phosphate-buffered saline (PBS) for 30 min at 37°C, and unlabeled fluorochrome was removed by two washes with sterile PBS. The optimum time for bacterial labeling was shown in the supplementary material (Figure S1A). The concentration of CFDA-SE

TABLE 1 Clinical feature of the healthy adults

Characteristic	Value	Reference interval
Total	131	-
Male	74 (56%)	-
Female	57 (44%)	-
Age (years ^a)	47 (24–85)	
White blood cells (10 ⁹ /L)	5.8 ± 1.21	3.5–9.5
Neutrophil (10 ⁹ /L)	3.39 ± 0.85	1.8–6.3
Glucose (mmol/L)	5.10 ± 0.69	3.92–6.16
ALT (U/L)	20.96 ± 12.08	9–50
AST (U/L)	23.98 ± 13.05	10–60
BUN (mmol/L)	4.86 ± 1.32	2.6–7.5
Scr (μmol/L)	75.16 ± 13.12	35–123

Data were shown as number (%) or mean ± SD.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Scr, serum creatinine concentration.

^aPresented as median and range.

was 10 μmol/L, and the volume ratio of CFDA-SE to bacteria was 2:1. The flow cytometer determines that the marking rate is greater than 95%, which is qualified and reserved. Due to the different cell wall structures, an additional 0.6% glutaraldehyde should be added to stain gram-negative bacteria.

2.3 | Phagocytosis assays

Fifty μL of heparinized whole blood and 20 μL of labeled bacterial suspension were added into test tubes, incubated for 45 min at 37°C in dark, and then placed on ice for 10 min to stop phagocytosis. RBCs were lysed using NH₄Cl lysing solution for 10 min. Finally, sterilized PBS was used for washing twice to remove excess bacteria. We conducted phagocytosis experiments at different time points and confirmed that 45 min was the optimal phagocytosis time. (Figure S1B). Phagocytosis of unlabeled bacteria under the same conditions was used as a negative control. To ensure neutrophil activity, all experiments were completed within 3 h after blood sample collection.

2.4 | Flow cytometric analysis

The phagocytic ability was evaluated in neutrophils, which was gated in the scatter diagram (FCS versus SSC), and its green fluorescence histogram (FL1) was analyzed. We collected 10,000–15,000 neutrophils per sample. We set the gate at the location with negative control of less than one percent. The results were expressed as a percentage of fluorescent cells in the total population studied (Figure 1). Flow cytometric enumeration was performed on Navios (Beckman Coulter), and the data were analyzed using Kaluza version

TABLE 2 The clinical data of the infected patients

Characteristic	Value	Reference interval
Total	38	-
Male	23 (60.5%)	-
Female	15 (39.5%)	-
Age (years ^a)	66 (31–88)	-
White blood cell (10 ⁹ /L)	9.97 ± 4.74	3.5–9.5
Neutrophils (10 ⁹ /L)	8.64 ± 4.72	1.8–6.3
CRP (mg/L)	71.25 (43.05–200.1)	0–10
PCT (ng/ml)	0.65 (0.20–3.96)	0–0.5
NLR	14.42 (5.78–21.18)	-
Types of infection		-
Gram-positive bacteria	5(13%)	-
Gram-negative bacteria	19(50%)	-
Mixed infection	14(37%)	-

Data were shown as number (%) or mean ± SD or median(Q2–Q4).

Abbreviations: CRP: C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; PCT, pro-calcitonin.

^aPresented as median and range.

2.0 software (Beckman Coulter). The detailed experimental process is shown by the flowchart (Figure 2).

2.5 | Statistical analysis

Statistical analysis was performed with GraphPad Prism version 8.0 software (GraphPad Software). Outliers were removed according to the Clinical and Laboratory Standards Institute (CLSI) C28-A3.¹¹

The data were checked with Kolmogorov-Smirnov test and found to be normally distributed. The normal distribution was expressed as mean ± standard deviation ($x \pm s$), $x \pm 1.96s$ was used as the reference interval, and t test was used for comparison. Those that do not conform to the normal distribution were represented by the median (quartile) [M (P25–P75)], 2.5–97.5 percentile nonparametric range was used as the reference interval,¹² and the comparison between the two groups was performed by the Mann-Whitney *U* test. The enumeration data were compared with the chi-square with Fisher's exact two-tailed *p*-value.

3 | RESULTS

3.1 | Reproducibility

The reproducibility of this method is important for clinical application. We use the coefficient of variation (CV) to evaluate reproducibility. The smaller the CV value, the better the repeatability. We therefore evaluated its reproducibility as follows: i) Phagocytosis test was conducted with heparinized whole blood from healthy adults according to the above standard procedure, and the phagocytosis rate of the sample was measured for these 20 times. The CV of phagocytic *Escherichia coli* and *Staphylococcus aureus* is 1.3% and 1.2%, respectively. ii) According to the above standard procedure, 20 phagocytosis experiments were conducted in healthy adults with heparinized whole blood, and the phagocytosis rates of these 20 samples were determined. The CV of phagocytic *Escherichia coli* and *Staphylococcus aureus* is 3.5% and 3.9%, respectively. The coefficient of variation (CV) was calculated by dividing the standard deviation by the mean, and the CV of each test was less than 5%. These data indicate that the reproducibility of the method is acceptable (Figure 3).

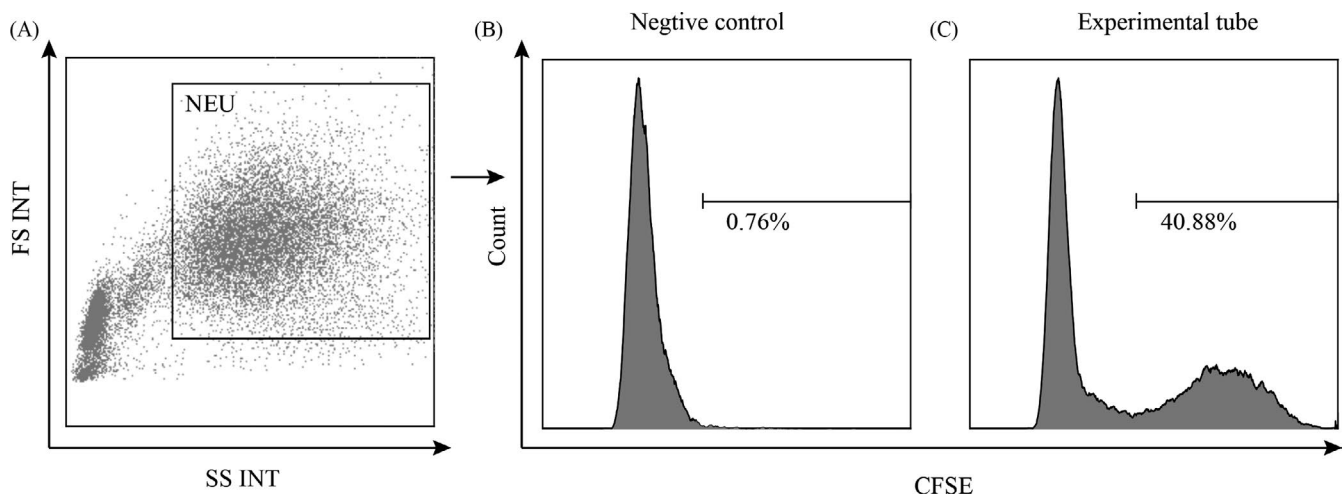


FIGURE 1 Gating strategy of neutrophils phagocytosis. A, Gated neutrophils in the scatter diagram (FS vs SS) were further analyzed for their phagocytic ability. B, The negative control was observed for neutrophils phagocytize unlabeled bacteria under the same experimental conditions. C, The increased percentages of fluorescence obtained by challenging neutrophils with CFSE-labeled bacteria were collected and calculated for phagocytosis percentages

FIGURE 2 Flow chart of experiment process

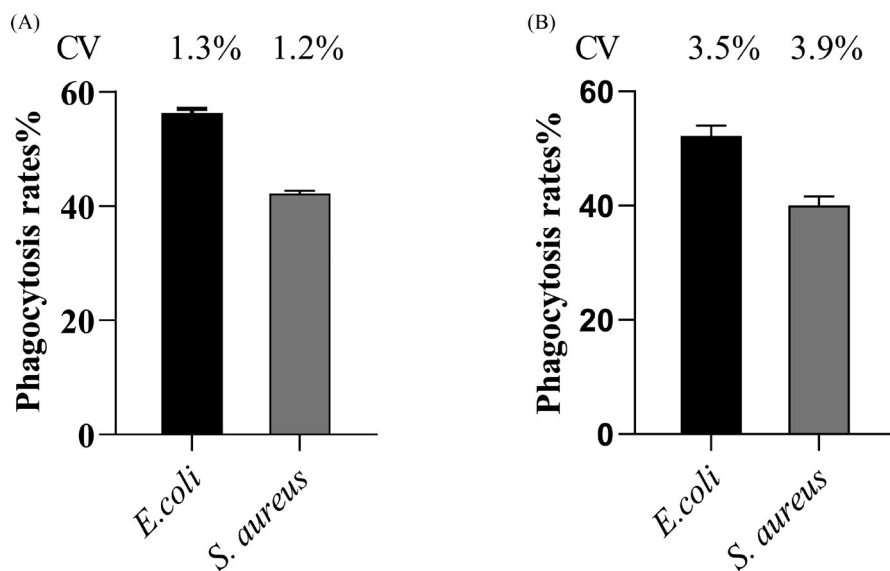
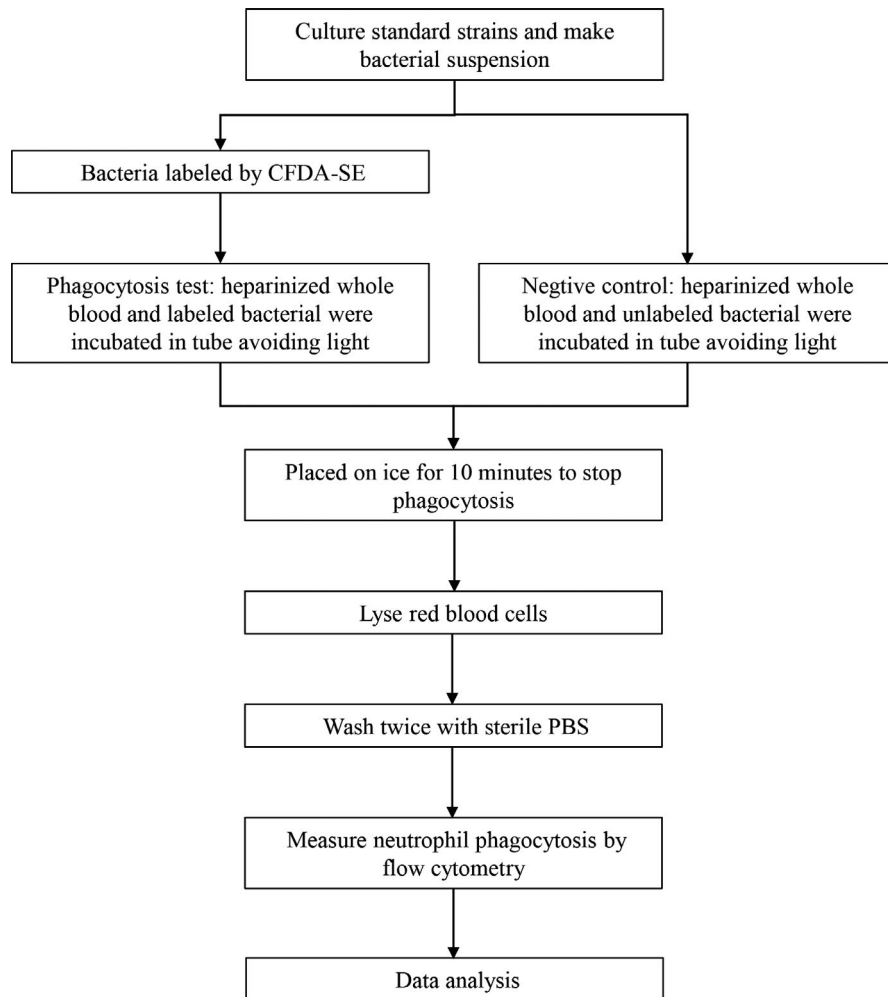


FIGURE 3 Evaluation of reproducibility of neutrophil phagocytosis by flow cytometry. A, The phagocytosis rate of heparinized whole blood of healthy people was determined 20 times according to the above standard procedure. B, Twenty phagocytic experiments were carried out in healthy adults with heparinized whole blood. The phagocytic rate of 20 samples was measured. Data are shown as mean \pm SD; CV, coefficient of variation.

3.2 | Establishment of the RIs of neutrophil phagocytosis

After removing outliers according to the requirements of CLSI C28-A3, the data were grouped and compared according to different

genders (male, female) or different ages (<60 years, \geq 60 years). Each group of data conforms to the normal distribution and homogeneity of variance. We observed that the phagocytosis rate of neutrophils to *Escherichia coli* or *Staphylococcus aureus* and in different age-groups or gender groups had no significant difference (Figure 4). Furthermore, the mean and RIs of whole blood neutrophil

phagocytosis were as follows: *Escherichia coli* (%):65.13(47.16–83.10); and *Staphylococcus aureus* (%):51.50 (33.16–69.57) (Table 3).

3.3 | Validation of the RIs of neutrophil phagocytosis in other healthy subjects and infected patients

To further validate the established RIs of neutrophil phagocytosis, we recruited another 37 healthy adults (19 males, 18 females) and 38 infected patients (23 males, 15 females) who were positive for bacterial cultures in their body fluids and/or blood. We observed that the phagocytic rates were significantly different in infected patients and healthy subjects. Besides, for the infected patients, 92.1% (35/38) and 50% (19/38) of results were out of *Escherichia coli*, and *Staphylococcus aureus* RIs of neutrophil phagocytosis, respectively; for healthy adults, 2.7% (1/37) and 5.4% (2/37) of results were out of *Escherichia coli*, and *Staphylococcus aureus* RIs of neutrophil phagocytosis, respectively (Figure 5). These data exhibited that the established RIs of neutrophil phagocytosis are reliable and suitable.

3.4 | Neutrophil phagocytosis was negatively correlated with the neutrophil count and neutrophil percentage

Because neutrophil phagocytic rates were significantly decreased in infected patients, we attempted to determine whether they are associated with classic inflammatory indices, including neutrophil count (NEU), percentage of neutrophils (NEU%), pro-calcitonin (PCT), C-reactive protein (CRP), and neutrophil-to-lymphocyte ratio (NLR) in infected patients. The phagocytosis rate of neutrophils of *Escherichia coli* and *Staphylococcus aureus* was negatively correlated with neutrophil count, neutrophil percentage, and NLR. (Table 4).

4 | DISCUSSION

Evaluation of neutrophil phagocytosis can provide the basis for the diagnosis, treatment, and prognosis of infectious diseases because

phagocytosis is one of the main anti-infective functions of neutrophils. Therefore, the traditional microscope detection method such as the microscopic method is complicated and volitional.

When a bacterial infection occurs, neutrophils migrate rapidly to the infected site, killing bacteria by phagocytosis, producing reactive oxygen species (ROS), and releasing neutrophil extracellular traps (NETs), etc., which can effectively prevent the further spread of infection.^{13,14} Neutrophil count and its percentage are still one of the most commonly used indicators for infection monitoring.¹⁵ Phagocytosis is one of the main anti-infective functions of neutrophils. Accurate evaluation of neutrophil phagocytosis can provide the basis for the diagnosis, treatment, and prognosis of infectious diseases. Therefore, the traditional microscope detection method is complicated and volitional, a simple, objective, and accurate assessment method is needed. Hence, we created a new method for the detection of whole blood neutrophil phagocytosis based on flow cytometry and established reference intervals.

Flow cytometry is a high-throughput assay developed in the past few decades. Flow cytometry has become an essential instrument for biomedical research and routine clinical detection of disease diagnosis, prognosis, and treatment monitoring.¹⁶ Recently, the detection of neutrophil phagocytosis by flow cytometry has been proved to be an efficient and objective method.^{10,17} Fluorescein isothiocyanate (FITC) or propidium iodide (PI) was used to label inactivated pathogens.¹⁸ Such methods will change the original biological characteristics of the pathogens and could not truly reflect the interaction between neutrophils and pathogens. Also, there were commercial kits that used pH-sensitive fluorescent dyes to label pathogens. The disadvantage was that the labeled pathogens were specific strains, which could not realize individual detection for different pathogen infections.¹⁹ CFDA-SE is a living cell fluorescent dye that could penetrate cell membranes and be catalyzed by intracellular esterases to decompose into CFSE, which can label lysine residues or other amino groups of intracellular proteins. The excitation wavelength is 488nm, and the emission wavelength is 518nm. Among them, gram-negative bacteria need to be stained with 0.6% glutaraldehyde.²⁰

Voyich et al.²¹ labeled *Staphylococcus aureus* from different sources with fluorescent dyes and observed that these bacteria showed significant differences in the process of phagocytosis by

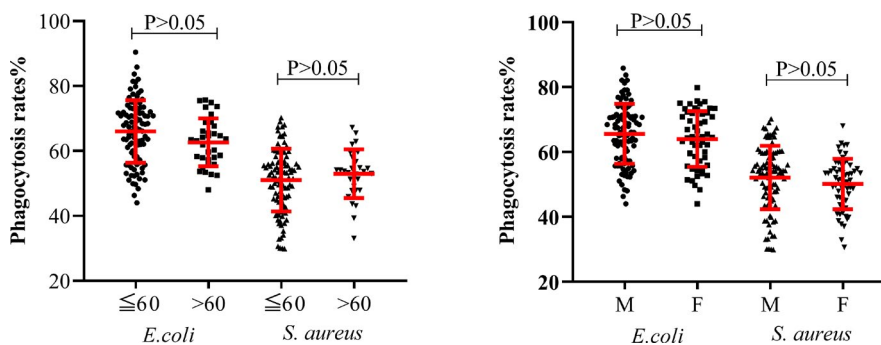


FIGURE 4 Differences in neutrophil phagocytosis between different genders or age groups. A, There was no significant difference in the phagocytosis rate of neutrophils to *E. coli* or *S. aureus* in different age groups. B, There was no significant difference in the phagocytosis rate of neutrophils to *E. coli* or *S. aureus* in different gender groups. *E. coli*, *Escherichia coli*. *S. aureus*, *Staphylococcus aureus*; ns, no significance

TABLE 3 The RIs of neutrophils phagocytosis

Bacterial species	N	Mean \pm SD(%)	RIs(%)
<i>Escherichia coli</i>	131	65.13 \pm 9.17	47.16–83.10
<i>Staphylococcus aureus</i>	131	51.50 \pm 9.22	33.43–69.57

neutrophils. Chatzimoschou's²² research pointed out that after using different antibiotics, the phagocytosis of bacteria by neutrophils has different degree changes. Our results suggest that neutrophil phagocytosis differs in healthy adults between *Escherichia coli* or *Staphylococcus aureus*. Therefore, when patients are infected with pathogenic bacteria, CFDA-SE-labeled bacteria can be used to detect the phagocytosis ability of neutrophils to this pathogen, to achieve the purpose of accurate evaluation of immune function and further study on the mechanism of action of antibiotics on the phagocytosis function of neutrophils.

For the convenience of clinical application, the whole blood sample was used in this study. Clawson et al.²³ conducted different gradient studies on the ratio of neutrophils to bacteria in the phagocytosis experiment, and the results showed that the ratio of neutrophils to bacteria could reach 1:400, and the number of bacteria phagocytes per neutrophil could reach up to 40. In our study, the ratio of whole blood to bacterial suspension was 5:2, which did not exceed the maximum phagocytosis ability of neutrophils described in the above study, enabling the number of bacteria to meet the phagocytosis of neutrophils without causing much background influence. Adhesion and phagocytosis were not strictly distinguished in this study. Because the phagocytic function of neutrophils is firstly reacted through adhesion, adhesion can be also regarded as part of the phagocytic function, and Hellebrekers et al.²⁴ observed through a laser scanning confocal microscope and found that few bacteria adhere to the surface of neutrophils. Therefore, it is considered that

bacteria adhered outside the cell membrane has little effect on the detection of flow cytometry.

In the study, it was also found that neutrophil phagocytosis in infected patients was significantly lower compared with healthy adults. Moreover, the neutrophils phagocytosis was negatively correlated with neutrophils count and neutrophils percentage. The possible causes are as follows: (i) Previous studies have shown that under normal circumstances, neutrophils spontaneously apoptosis at 24 h after differentiation and maturation. However, their life span is prolonged during infection, which makes neutrophil easier to adapt to the inflammatory environment and enhances their ability to affect surrounding cells, which in turn increases the number and proportion of circulating granulocytes.^{25,26} (ii) Inflammation will mobilize the bone marrow hematopoietic function, resulting in the increase in neutrophilic band form in peripheral blood and even myelocyte and metamyelocyte. However, the increased part of the immature cells does not have a normal function, thus reducing the proportion of neutrophils with phagocytosis.²⁷

Morris et al.²⁸ showed that impaired neutrophil phagocytosis is an independent risk factor for ICU acquired infection. Some studies have shown that neutrophil phagocytosis is closely related to the development and prognosis of some infectious diseases.²⁹ Pinder et al.³⁰ demonstrated that the administration of GM-CSF (granulocyte-macrophage colony-stimulating factor) *in vivo* can improve the neutrophil phagocytosis and clinical outcomes such as survival rate and hospital length of stay. The measurement of neutrophils phagocytosis may have a good application prospect for evaluating the prognosis of patients.

One limitation of this study is that it is a single-center study. Multi-center research and more research objects will make the reference interval more scientific and universal. Additionally, the neutrophil phagocytosis to different types of bacteria was not

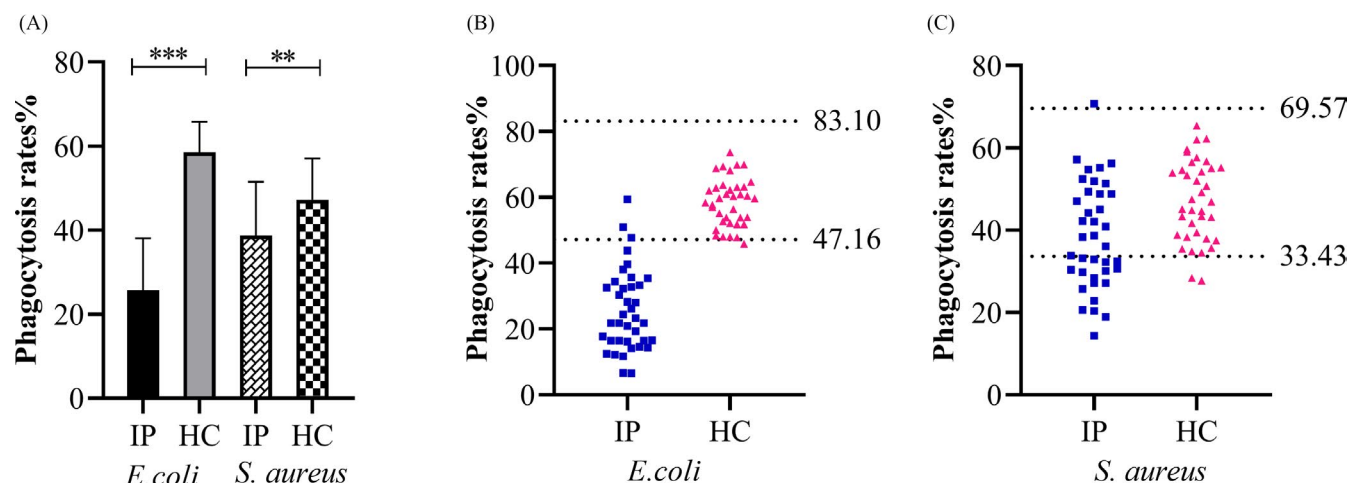


FIGURE 5 Validation of the RIs of neutrophil phagocytosis in other healthy subjects and infected patients. A, Neutrophil phagocytosis was significantly different in healthy adults and infected patients. B, Phagocytosis of neutrophils to *E. coli* in healthy adults and infected patients. C, Phagocytosis of neutrophils to *S. aureus* in healthy adults and infected patients.***, $p < 0.0005$;**, $p < 0.005$; HC, healthy control; IP, infected patients. *E. coli*, *Escherichia coli*. *S. aureus*, *Staphylococcus aureus*

TABLE 4 Correlation analysis between phagocytic rate and classical indicators

	Correlation		
	R	95% CI	P
<i>Escherichia coli</i>			
NEU ($\times 10^9/L$)	-0.379	-0.623 to -0.0677	0.0190 [*]
NEU%	-0.442	-0.667 to -0.142	0.006 [*]
CRP (mg/L)	-0.0590	-0.372 to 0.266	0.725
PCT (ng/ml)	-0.223	-0.506 to 0.105	0.179
NLR	-0.318	-0.586 to 0.0114	0.0515
<i>Staphylococcus aureus</i>			
NEU ($\times 10^9/L$)	-0.522	-0.721 to -0.242	0.000 [*]
NEU%	-0.361	-0.617 to -0.0374	0.0258 [*]
CRP (mg/L)	0.0437	-0.280 to 0.358	0.795
PCT (ng/ml)	-0.152	-0.449 to 0.176	0.825
NLR	-0.327	-0.592 to 0.00137	0.0449 [*]

* $p < 0.05$.

completely consistent, the reference interval investigation of Gram-positive bacteria and Gram-negative bacteria was carried out, but the reference interval investigation of fungal was lacking.

5 | CONCLUSION

We developed an objective, simple, rapid, and accurate method for the evaluation of phagocytosis and established the RIs of whole blood neutrophils for *Escherichia coli* and *Staphylococcus aureus*, respectively. Additional clinical studies are needed to verify these reference intervals and to establish the reference intervals of whole blood neutrophils for fungi that were not available in this study.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

Yonglie Zhou conceived of the study and was responsible for data analysis and interpretation. Fangfang Yang drafted the manuscript. Liping Yang interpreted the patient data. Fangfang Yang, Haoran Li and Fujie Zhang performed the experiments and analyzed the data. All the authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

Data and materials associated with this work are available by request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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