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CD35 and CD64 of Neutrophils Can Differentiate Between Bacterial and Viral Infections in Children by Simultaneous Quantitative Analysis

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: The aim of this study was to determine the relationship between the expression of CD35 and CD64 from white blood cells (neutrophil, monocytes, and lymphocytes) and acute infectious diseases in children.

Material/Methods: The blood samples were collected from 104 children with infections (42 viral infections and 62 bacterial infections). Blood samples were stained with CD45-PC5, CD35-FITC, and CD64-PE, and the fluorescence intensities were measured by flow cytometer, and then the ratio of CD35 to CD64 was calculated.

Results: The ratio of CD64/CD35 on neutrophils (NCD35/NCD64) was significantly different between the bacterial group, the virus group, and the healthy control group. According to receiver operating characteristic (ROC) analysis, a cutoff value of 7.256 (sensitivity: 90.0%, specificity: 93.7%) was determined for the NCD35/NCD64 ratio.

Conclusions: This study shows that NCD35/NCD64 is helpful in the differential diagnosis of acute viral infection and bacterial infection in children.

MeSH Keywords: Acute Disease • Brain Injuries • Neutrophils

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Background

In children, a variety of pediatric acute infectious diseases are the leading cause of morbidity and mortality, and results in enormous social and economic burdens. However, it is often difficult to distinguish between bacterial infections and viral infections in clinical practice, because children cannot accurately express the disease, and most laboratory tests cannot accurately distinguish between the 2 infections, or need several days to determine (such as microbial culture), or can only diagnose a single pathogen (such as various serological antibody detection and polymerase chain reaction detection of nucleic acids). This results in a lot of prescriptions of antibiotics that are used without an accurate distinction between various causes of infection. Potential antibiotic abuse is considered a serious threat to mankind. Therefore, there is a need for a new method of distinguishing between viral and bacterial infections. Quantitative analysis of leukocyte surface receptors is a candidate method to research.

FcγRI (CD64) in neutrophils is used to assess infections, particularly bacterial infections [1,2]. One study found that neutrophil CD64 expression was not significantly different from a control group in patients with rheumatoid arthritis without acute infection [3]. Therefore, neutrophil CD64 might be able to identify whether a patient with an inflammatory disease has a bacterial infection. A few studies have supported the use of granulocyte CD64 to distinguish between bacterial infections and rheumatoid arthritis [4]. In addition, complement receptor 1 (CR1/CD35) is another potential bacterial infection marker for neutrophils, which shows higher sensitivity and specificity in distinguishing between bacterial and viral infections [5]. Several research studies have shown that CD35 can distinguish between bacterial and viral infections [6]. Another report found that CD64 and CD35 can distinguish among bacterial infections, viral infections, and inflammatory diseases by simultaneous measurement [7].

Therefore, this study aimed to detect the expression of CD64 and CD35 in children with acute infectious diseases, and distinguish the bacterial and viral infections by these aforementioned markers.

Material and Methods

Research participants

A total of 138 children with suspected infections admitted to hospital from April to December 2017 in Tongde Hospital of Zhejiang Province were selected, and 35 children with health checkups were selected for blood test, too. After clinical and laboratory, or imaging examinations, 104 children (54 males and 48 females) with an average age of 5±2.1 years were diagnosed as having bacterial or viral infections. The bacterial infection group (62 cases) and the virus infection group (42 cases) included cases confirmed by microbial culture and clinical diagnosis (Table 1). Antibiotics used in this study population after admission included ceftriaxone, ceftazidime, deoxycephalosporin, piperacillin sulbactam sodium, and amoxicillin clavulanate potassium. The study has been reviewed by the TongDe Hospital of Zhejiang Province Hospital Ethics Committee, and informed consent was obtained from participating patient's family.

Table 1. Diagnosis and pathogens in children.

		Number of cases	Diagnosis (number of cases)	Pathogen (number of cases)
Bacterial infection group	Microbiologically diagnosed bacterial infection group	25	Bacterial pulmonary infection (15) Suppurative tonsillitis (5) Urinary tract infection (3) Sepsis (2)	*Streptococcus pneumoniae* (9) *Klebsiella pneumoniae* (7) Type B Hemolytic Streptococcus (4) *Escherichia coli* (3) *Staphylococcus aureus* (2)
	Clinically diagnosed bacterial infection group	37	Bacterial pulmonary infection (19) Suppurative tonsillitis (16) Urinary tract infection (2)	
Virus infection group	Microbially diagnosed virus infection group	18	Upper respiratory tract infection (9) Infectious mononucleosis (9)	Epstein-Barr virus (9) Adenovirus (6) Influenza A virus (2) Respiratory syncytial virus (1)
	Clinically diagnosed viral infection group	24	Infectious mononucleosis (11) Upper respiratory tract infection (7) Herpetic angina (6)	

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The definition of microbial-diagnosed bacterial infections included: a positive culture from blood or urine a child with urinary tract infections, the number of pathogens was greater than 10^5/mL, and throat swabs or sputum in children with respiratory infections.

The definition of a virus-infected case of microbial diagnosis included: a positive IgM antibody in the serum of the child or a doubling of the IgG antibody in the serum (n=14), or a viral nucleic acid detected by polymerase chain reaction.

According to the patient's symptoms and signs, combined with the clinical course, the clinically diagnosis of bacterial and viral infections were mainly classified by the attending physician, and the final diagnosis of the empirical treatment effect was observed.

Exclusion criteria was as follows: 1) child had no explicitly diagnosis or transferred; 2) used antibiotics before admission; 3) concomitant with other chronic infectious diseases (such as hepatitis B, hepatitis C, etc.) or autoimmune diseases; 4) had congenital diseases (congenital heart disease, type 1 diabetes, etc.) or congenital immunodeficiency.

Research methods

Blood samples were used in clinical trials and for biomarker testing. Bacterial infection was identified by blood culture and sputum culture. From each test, 1 mL of EDTA-K_2 anticoagulation blood was collected. First, an isotype control was set up, and 50 U of whole blood was added to CD45-PC5 (clone A07785), Ig-G-FITC (clone number A07795), and Ig-G-PE (clone MOPC-21) at 20 uL each. CD45-PC5 was purchased from Beckman Coulter; Ig-G-FITC and Ig-G-PE was purchased from BioLegend. Samples were protected from light for 20 minutes, then hemolysis solution of 300 mL was added, then after 20 minutes, the samples were centrifuge at 2000 rpm for 5 minutes. The supernatant was discarded, and 200 mL of the dilution solution was added. A Beckman Coulter Navios flow cytometry was used to detect the expression curve of FITC and PE. After setting the negative limit, the expression levels of CD35 and CD64 were detected in the samples.

Whole blood (50 U) was added to CD45-PC5 (clone A07785), CD35-PE (clone E11), and CD64-PE (clone PNIM1604U) each at 20 uL; and the antibodies IgG1-PE(MOPC-21) and IgG1-FITC(A07795), which were purchased from Beckman Coulter Company, were added. The samples were protected from light for 20 minutes, and hemolytic agent, 300 mL, was added, then after 20 minutes, the samples were centrifuge at 2000 rpm for 5 minutes, the supernatant was discarded, and 200 mL of the dilution solution was added, and the mean fluorescence intensity was measured. Data collected included: NCD35 as the average fluorescence intensity of CD35 of neutrophils; NCD64 as the average fluorescence intensity of CD64 of neutrophils; NCD35/NCD64 as the ratio of the average fluorescence intensity of CD35 to CD64 of neutrophils; MCD35 as monocyte. The average fluorescence intensity of CD35; MCD64 as the average fluorescence intensity of CD64 of monocytes; MCD35/MCD64 as the ratio of the average fluorescence intensity of CD35 to CD64; LCD35 as the average fluorescence intensity of CD35 of lymphocytes; LCD64 as the average of C64 of lymphocytes. For fluorescence intensity, LCD35/LCD64 has a ratio of the average fluorescence intensity of lymphocytes CD35 to CD64.

Statistical analysis

The significance of the differences was determined by the Kruskal-Wallis test among the 3 groups. The significance of the difference was determined by the Mann-Whitney U test between 2 groups, and $P<0.01$ was defined as the level of significance. To determine the most diagnostic value of the indicator and to determine the cutoff, a receiver operating curve (ROC) analysis was used. The statistical analysis software is SPSS 19.0.

Results

Expression of CD35 and CD64 in different groups

The average fluorescence intensity levels of neutrophils CD35 (NCD35) and CD64 (NCD64), and the ratio of CD35 and CD64 of the 3 cells (NCD35/NCD64, MCD35/MCD64, and LCD35/LCD64) were measured in the bacterial infection group. There was statistical significant difference between the virus-infected group and the healthy control, $P<0.01$. Furthermore, the Mann-Whitney U test was used for the pairwise comparison. For NCD35, only the bacterial group and the virus group were statistically different, but the bacterial group did not differ from the healthy control group. For NCD64, only the virus group was significantly different from the healthy control group. For MCD35/MCD64 and LCD35/LCD64, the bacterial group and the virus group were different from the healthy group, but there was no difference between the bacterial group and the virus group. Only for NCD35/NCD64, was the bacterial group and the virus group significantly different from the healthy group, and there was also a statistical difference between the bacterial group and the virus group, $P<0.01$ (Table 2, Figure 1). This suggested that NCD35/NCD64 was identified as a good marker of bacterial infections and viral infections.

Operating curve (ROC) analysis

To determine the most diagnostically valuable markers and to determine the cutoff value for identifying bacterial infections

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Table 2. Differences in expression of CD35 and CD64 between neutrophils, monocytes and lymphocytes in the bacterial infection group, the virus infection group and the healthy control group. Raw data are mean fluorescence intensity MFI mean ±SD. The three groups of data were compared using the Kruskal-Wallis test ($p<0.01$), and the pairwise data were compared using the Mann-Whitney U test ($p<0.01$).

	Bacterial infection group A	Virus infected group B	Health control group C	*p* Value Kruskal-Wallis test	*P*-value Mann-Whitney U test		
					A *vs.* B	A *vs.* C	B *vs.* C
NCD35	17.31±1.200	11.20±1.498	15.09±1.518	0.007	0.002	0.629	0.023
NCD64	1.862±0.183	3.176±0.591	0.9756±0.173	0.001	0.025	0.010	0.000
NCD35/NCD64	11.80±1.437	4.275±0.488	19.34±3.005	0.000	0.000	0.005	0.000
MCD35	21.53±1.768	14.99±1.662	18.62±1.890	0.095	0.035	0.719	0.148
MCD64	6.369±0.555	6.405±0.855	3.693±0.381	0.021	0.874	0.004	0.034
MCD35/MCD64	3.832±0.312	2.725±0.348	5.589±0.762	0.001	0.017	0.009	0.000
LCD35	4.914±0.307	3.548±0.545	5.867±0.650	0.012	0.014	0.314	0.008
LCD64	0.4969±0.033	0.5521±0.103	0.3549±0.033	0.021	0.858	0.011	0.007
LCD35/LCD64	10.95±0.847	7.877±1.463	17.15±1.895	0.001	0.024	0.004	0.001

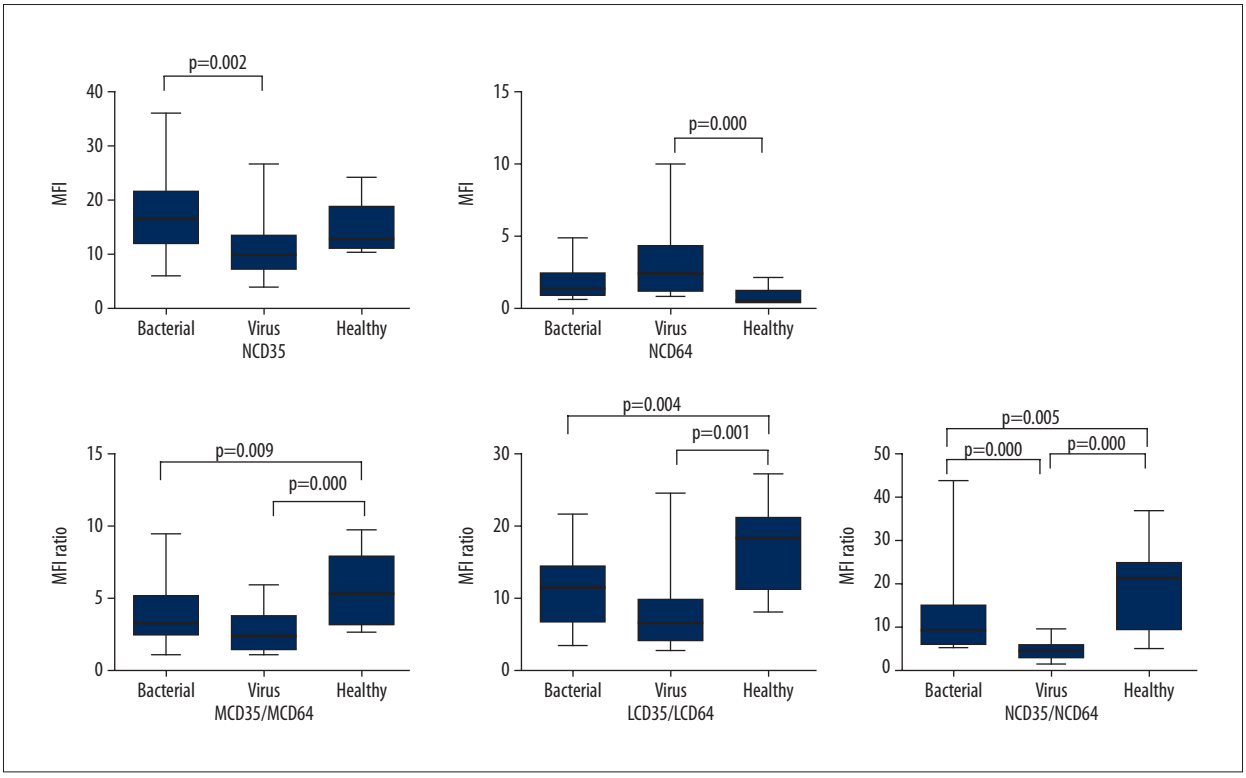

Figure 1. Comparison of NCD35, NCD64, NCD35/NCD64, MCD35/MCD64, and LCD35/LCD64 expression levels in the bacterial infection group, the virus infection group, and the healthy control group. In the box plot data, the lines within the box represent the median value. The boxes show 25% and 75%, and the bars represent the maximum and minimum values, using the Mann-Whitney U test (*P*<0.01).

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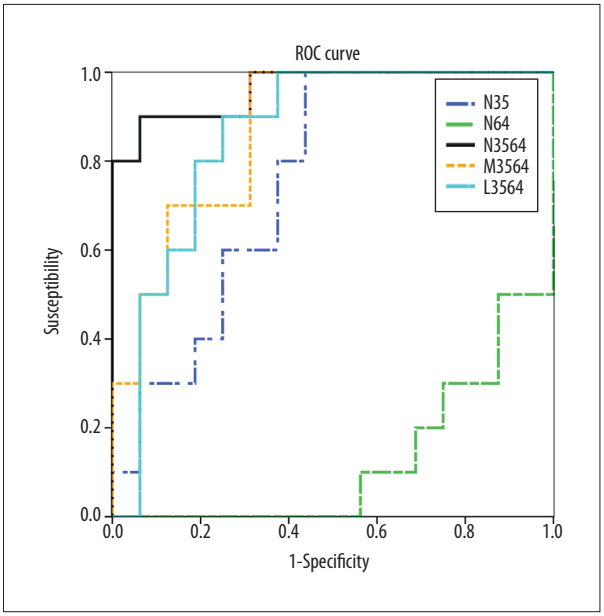

Figure 2. Analysis of receiver operating characteristic curves when diagnosing viral infections.

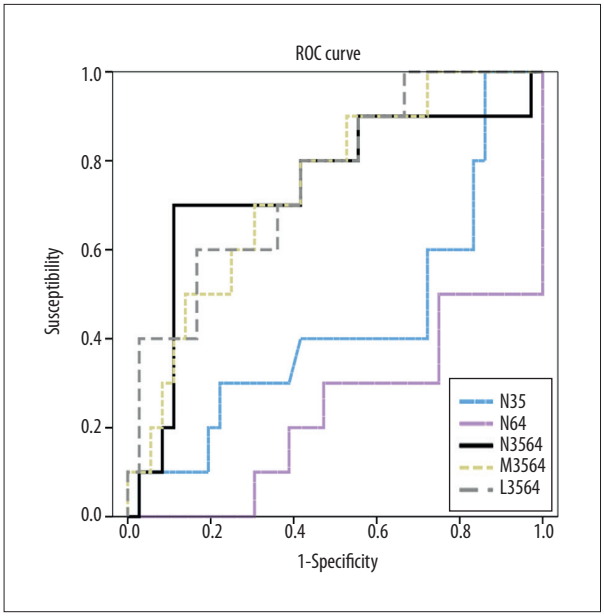

Figure 3. Receiver operating characteristic curve analysis for the diagnosis of bacterial infections.

Table 3. Diagnostic area under the viral infection curve (AUC), p-value, 95% confidence interval (CI).

	Area under the curve (AUC)	*p* Value	95% confidence interval (CI)	
			Lower limit	Upper limit
NCD35	0.756	0.031	0.572	0.941
NCD64	0.125	0.002	0.000	0.257
NCD35/NCD64	0.963	0.000	0.893	1.000
MCD35/MCD64	0.869	0.002	0.733	1.000
LCD35/LCD64	0.856	0.003	0.708	1.000

Table 4. Diagnostic bacterial infection curve area (AUC), p-value, 95% confidence interval (CI).

	Area under the curve (AUC)	*p* Value	95% confidence interval (CI)	
			Lower limit	Upper limit
NCD35	0.432	0.514	0.219	0.645
NCD64	0.233	0.011	0.054	0.413
NCD35/NCD64	0.739	0.022	0.543	0.934
MCD35/MCD64	0.739	0.022	0.574	0.904
LCD35/LCD64	0.758	0.013	0.593	0.923

and viral infections we used the ROC curve for analysis, including NCD35, NCD64, NCD35/NCD64, MCD35/MCD64, and LCD35/LCD64, and the results suggested that the area under the curve (AUC) for NCD35/NCD64 was 0.963 (95% confidence interval [CI] 0.893–1.000, *P*=0.000) when there was virus infection (Figure 2, Table 3). According to the ROC curve evaluation, NCD35/NCD64 had more diagnostic value; the cutoff value was 7.256. The diagnostic virus infection sensitivity was 0.900, and the specificity was 0.937.

The ROC curve analysis at the time of bacterial infection showed that the AUC of the LCD35/LCD64 was 0.758 (95% CI: 0.574–0.904, *P*=0.013) (Figure 3, Table 4). According to the ROC curve evaluation, NCD35/NCD64 had an AUC of 0.739, a cutoff

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value of 18.264, a sensitivity of 0.700, and a specificity of 0.889; whereas, LCD of LCD35/LCD64 had an AUC of 0.758, a cutoff of 15.878, a diagnostic bacterial infection sensitivity of 0.600, and a specificity of 0.833. Although the AUC of LCD35/LCD64 was larger than NCD35/NCD64, the former showed no significant difference between the bacteria group and the virus groups in the previous statistical analysis. Similarly, MCD35/MCD64 was not significantly different between the bacteria group and the virus group. Therefore, we suggest that both should not be chosen as diagnostic indicators.

Discussion

In this study, the average fluorescence intensity of CD35 and CD64 on neutrophils, monocytes, and lymphocytes was detected by flow cytometry. The Kruskal-Wallis test revealed that NCD35, NCD64, NCD35/NCD64, MCD35/MCD64, and LCD35/LCD64 had significant differences between the bacterial group, the virus group, and the healthy group. By Mann-Whitney U test, only NCD35/NCD64 was found to be significantly different among the 3 groups, suggesting that NCD35/NCD64 might be an effective diagnostic marker to distinguish the bacterial and viral infections. To determine the diagnostic potency, the ROC curve analysis showed that the sensitivity of NCD35/NCD64 in the diagnosis of viral infection was 0.900, the specificity was 0.937, and the cutoff value was 7.256. In the diagnosis of bacterial infection, the sensitivity of NCD35/NCD64 was 0.700, the specificity was 0.889, and the cutoff value was 18.264. When NCD35/NCD64 was less than 7.256, the possibility of viral infection was large. When it was greater than 7.256 and less than 18.264, the possibility of bacterial infection was large. However, since the diagnosis of bacterial infection in NCD35/NCD64 was less than 90% of the AUC area and the diagnostic performance was poor, it is recommended to be used to assist other biomarkers in diagnosis. Previous studies have used a single CD35 or CD64 as the research object, and its correlation with bacterial or viral infection has been reported to be high and low respectively, and the sensitivity and specificity were also quite different [8]. Another study on the ratio of CD35/CD64 was reported to have 100% sensitivity and 86% specific for patients with rheumatoid arthritis. The sensitivity of diagnosis of bacterial infection was 67% and the specificity was 80% [9], and the results are in consistent with the present study.

CR1/CD35, MCP/CD46, GPI, and DAF/CD55 are both complement activation regulator (RCA) protein families [10,11]. CD55 inhibits the activation of C3 and C5 by accelerating the decay of C3 and C5 convertase formation. CD46 modulates C3 activity by cleavage of C3b by cofactor proteins. The CD35 indicates the activity of both CD46 and CD55 [12]. CD35 is only weakly expressed on the surface of resting neutrophils, mainly stored in intracellular particles [13]. In resting monocytes, CD35 is stored in secretory vesicular granules in bacterial infections, exposed to pro-inflammatory cytokines, neutrophils, and monocytes can rapidly remove intracellular particles, granules, and cytoplasm. Fusion of the membrane leads to upregulation of CD35 on the cell surface [14]. CD64, a relatively classic indicator of inflammation, is a high-affinity immunoglobulin FCGR1 [15]. However, it is upregulated in the immune response caused by pro-inflammatory cells [14]. Upregulation of CD64 in neutrophils is affected by interferon C (IFN-C) and granulocyte colony-stimulating factor (G-CSF) [16,17] while CD35 expression is regulated by granulocyte macrophage colony-stimulating factor and tumor necrosis factor alpha (TNF-alpha), but not by IFN-C and G-CSF [18,19]. The regulatory mechanisms are different, which may be related to the difference in expression levels of CD35 and CD63 after bacterial or viral infection.

Conclusions

The ratio of the neutrophil CD35/CD64 can be used to distinguish acute viral and bacterial infections in children. When NCD35/NCD64 is less than 7.256, the possibility of viral infection is large. When it is greater than 7.256 and less than 18.264, it might indicate a bacterial infection. However, because the AUC area of NCD35/NCD64 in the diagnosis of bacterial infection was less than 90%, the diagnostic performance was not good; thus, it is recommended to be used to assist other biomarkers in diagnostic testing. This study can help pediatricians make accurately early diagnose and avoid overuse of antibiotics.

Conflict of interest

None.

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