Original Article

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CD64 Expression by Neutrophils as a Diagnostic Marker for Sepsis: A Critical Evaluation

Pathology Section

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ABSTRACT

Introduction: The increasing incidence of sepsis and its associated mortality has urged the search for rapid and reliable diagnostic test. Flow cytometry offers rapid assessment of change in antigen expression by neutrophils in blood in suspected sepsis cases. An increased expression of CD64, a high affinity opsonic surface receptor, by neutrophils is seen in sepsis.

Aim: To critically analyse the role of neutrophilic CD64 expression by different possible flow cytometric parameters in sepsis.

Materials and Methods: This case-control study was conducted in Department of Pathology of a Sri Guru Ram Rai Institute of Medical and Health Sciences, Dehradun, Uttarakhand, India from February 2017 to July 2019. It included peripheral blood samples from 43 cases of sepsis and their corresponding age matched controls. Flow cytometric analysis of blood samples for CD64 expression by neutrophils was done. The statistical analysis was performed with Statistical Package for Social Sciences (SPSS) version 18.0, Chicago, IL.

Results: Flow cytometric analysis revealed a statistically significant increased expression of CD64 by neutrophils in sepsis patients, in terms of all four parameters analysed- increased expression of CD64 as compared to controls, in terms of percentage of neutrophils expressing CD64 (63.88±34.12 in cases), mean Fluorescent Intensity (FI) (2137.19±2319.71), Median Fluorescent Intensity (MFI), (2011.28±2261.89) as well as Neutrophil:Lymphocyte (N:L) CD64 index (7.29±9.66). Sensitivity and specificity of each parameter evaluated in present study varied from 60.4-67.4% and 81.4-83.7%, respectively.

Conclusion: Quantitative expression of neutrophil CD64 (nCD64), by flow cytometry, in terms of percentage expression, MFI, mean fluorescent intensity and Neutrophils:Lymphocytes CD64 index is helpful in diagnosing sepsis patients even after 72 hours of onset. This is a rapid, reliable and cost-effective investigation. Hence, enabling prompt and judicious treatment of sepsis.

Keywords: Flow cytometry, Mean fluorescent intensity, Median fluorescent intensity

INTRODUCTION

'Sepsis', derived from greek word 'sipsi' which implies "make rotten", was introduced by Hippocrates. The term 'Sepsis' is applicable, when an infectious aetiology is proven or suspected and the response results in damage to uninfected organs [1]. In 1992, the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) introduced definitions for Systemic Inflammatory Response Syndrome (SIRS) and sepsis [2]. In 2016, sepsis was re-defined as life-threatening organ dysfunction that is caused by a dysregulated host response to infection without the requirement of SIRS criteria [3]. Every year, 18 million new cases of sepsis are being reported worldwide with mortality ranging from 30-50%. In India, 28.3% patients in Intensive Care Unit (ICU) develop sepsis with a mortality rate of 39% [4]. Three million newborns and 1.2 million children are diagnosed with sepsis globally per year [5].

Prompt diagnosis and appropriate antimicrobial therapy are crucial for decreasing sepsis related mortality. The irony is that the diagnosis of sepsis is as controversial and complicated as is its definition. Many biomarkers like Procalcitonin (PCT) and C-Reactive Protein (CRP) have been evaluated for the same with variable outcomes. Blood culture and other haematological and serological tests are currently being used, but these have their own limitations e.g., increased turnaround time especially for the gold standard blood culture or lack of specificity in case of certain serological markers. As opposed to these investigations, flow cytometry offers a fast, reproducible and cost-effective modality, if any suitable immunophenotypic marker in peripheral blood cells proves to be specific for the diagnosis of sepsis.

Till date, flow cyometry has rendered possible to assess earliest changes in antigens expressed by inflammatory cells rapidly and accurately. Amongst the various antigens evaluated for diagnosis and prognosis of sepsis cases, CD64 expression by neutrophils (nCD64) seems promising with a high sensitivity and specificity. Quiescent neutrophils express low-affinity receptors for Immunoglobulin G (IgG), FcγRII (CD32) and FcγRIII (CD16), but not the high-affinity opsonic receptor FcγRI (CD64). In recent years, various studies have shown increased expression of nCD64 measured by flow cytometry in cases of sepsis [6-8]. Current study was conducted to critically analyse all possible flow cytometric parameters of measuring neutrophilic CD64 expression in sepsis and also to assess its utility practically.

MATERIALS AND METHODS

This case-control study was conducted in Department of Pathology, Sri Guru Ram Rai Institute of Medical and Health Sciences, Dehradun, Uttarakhand, India, i.e., from February 2017 to July 2019. Approval from the Institutional Ethical Committee was taken (Reference number: SGRR/IEC/16/18). The study was conducted in accordance to the Declaration of Helsinki.

A total of 43 cases who were admitted in the Intensive Care Department of the institute were enrolled in this study. Written informed consent was taken from all subjects (cases as well as controls).

Inclusion criteria

For adults: Patients with strong suspicion of infection and any of the two that is fever, heart rate (>90 beat per minute) or increased respiratory rate (>20 per minute) and abnormal white blood cell count (>12,000/μL/<4000/uL or 10% band forms) [2] were included.

For Neonates: Cases were included according to European Medicines Agency (2010) criteria for sepsis [9].

Exclusion criteria: Cases with immunocompromised status and in which no clear separation between neutrophils and monocytes was

possible on CD45 versus Side Scatter (A) plot during flow cytometric analysis were excluded from the study.

Selection of controls: For every case included in the study, an age matched healthy individual (without sepsis) was also enrolled as control. They were either the guardian or the subjects who visited the Outpatient Department (OPD). A total of 43 controls were included in the study.

Procedure

Blood samples (5 mL) were collected to assess Complete Blood Counts, C-Reactive Protein (CRP), Procalcitonin (PCT), blood culture and flow cytometric analysis for CD64 expression. Antibiotics were administered to all patients as per institutional protocol.

Flow cytometric analysis: For flow cytometry, 50 microlitre Ethylenediamine Tetraacetic Acid (EDTA) samples from all cases and controls were processed within 24 hours with stain-lyse-wash method. V500c labeled CD45 and Fluorescein Isothiocyanate (FITC) labeled CD64 antibodies used in the study were validated and titrated. The antibodies, Flourescence-Activated Cell Sorting (FACS) lyse solution, and Perm II permeabilisation buffer were obtained from Becton Dickinson Biosciences (BD), USA.

The samples were then analysed on BD FACS Canto II, eight color flow cytometer using FACS Diva software. CST beads were run as quality check daily. Neutrophils and lymphocytes were gated on CD45 versus Side Scatter (A) plot. A sample was included in the study, only if the neutrophil and monocyte populations could be clearly identified. CD64 expression was assessed in samples from both controls and cases by studying the following four parameters:

a) Percentage of neutrophils expressing CD64, by using quadrant gates.

b) Median Fluorescent Intensity (MFI) FITC CD64 of neutrophils.

c) Mean fluorescent intensity (FI) FITC CD64 of neutrophils.

d) MFI FITC CD64 neutrophils/MFI FITC CD64 lymphocytes ratio i.e., Neutrophil:Lymphocyte (N:L) CD64 index.

STATISTICAL ANALYSIS

The values of each parameter were compared between controls and patients for any statistical significance. Chi-square test was applied. The p-value <0.05 was considered statistically significant. The sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of each parameter was calculated. The results of the four parameters were also assessed for any association with CRP, PCT and culture studies, where available. The statistical analysis was performed with Statistical Package for Social Sciences (SPSS) version 18.0, Chicago, IL.

RESULTS

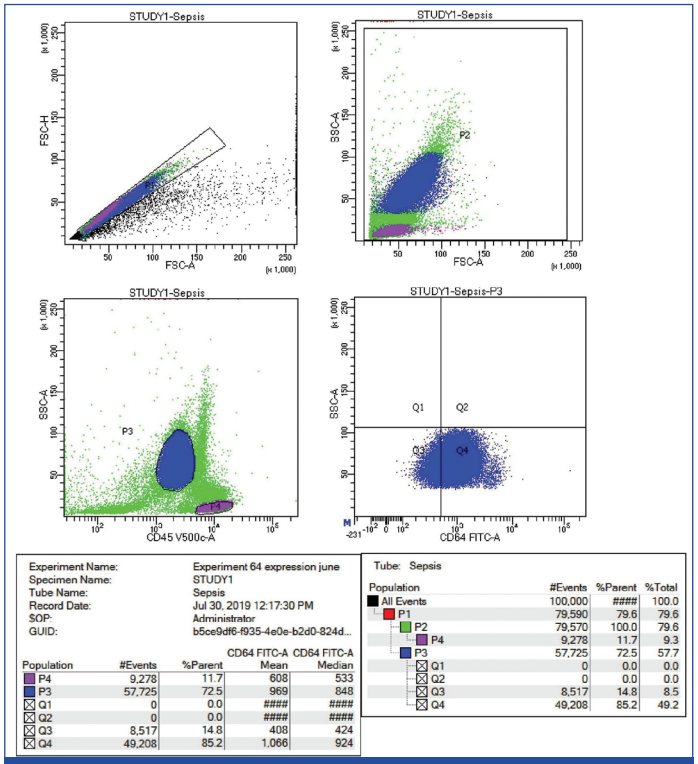
A total of 86 blood samples were received, 43 samples from sepsis patients and 43 from controls. Majority of patients with sepsis in this study were in the age group 18-40 years. All the neonates in the present study were less than 7 days old. A slight male predominance 22 (51.2%) was seen in the study population [Table/Fig-1].

Age group	Cases		Controls	
	Male n=22	Female n=21	Male n=22	Female n=21
<7 days	3	1	3	1
18-40 years	10	11	10	11
41-60 years	3	5	3	5
>60 years	6	4	6	4

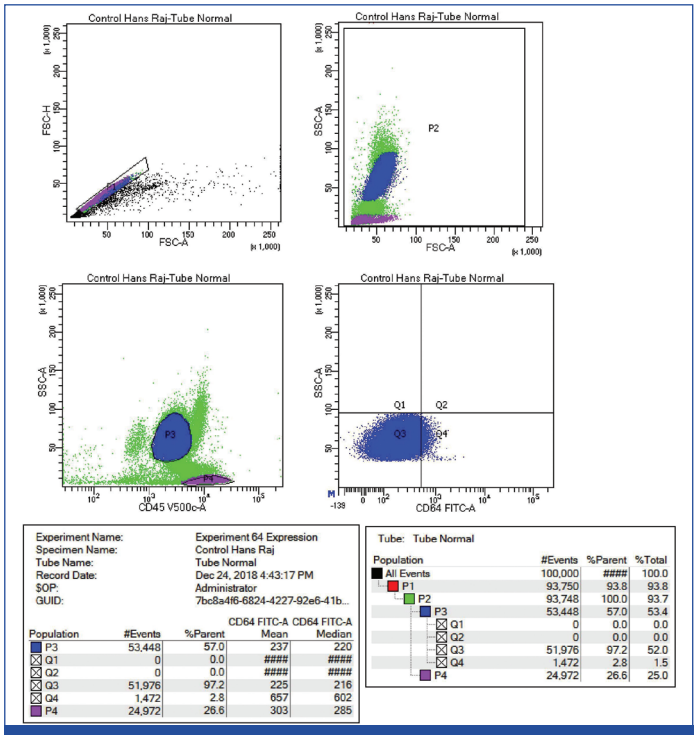
[Table/Fig-1]: Age wise distribution of the study population N=43.

Majority of the samples, 35 (81.39%) were collected after 72 hours of admission, while 6 (13.95%) samples were collected between 24-72 hours and only 2 (4.65%) were collected within 24 hrs. All patients had received antibiotics prior to sampling.

CD64 expression was found to be higher in terms of all four parameters assessed in sepsis patients than controls and this difference was found to be statistically significant [Table/Fig-2a,b,3].



[Table/Fig-2a]: Sepsis cases: 85.2% of neutrophils express CD64 with a Median Fluorescent Intensity (MFI) of 848 and Mean Fluorescent Intensity of 969.



[Table/Fig-2b]: Control: 2.8% of neutrophils express CD64 with a MFI of 220 and Mean Fluorescent Intensity of 237.

Parameters	Cases (Mean±SD)	Controls (Mean±SD)	p-value
Percentage of neutrophils expressing CD64	63.88±34.12	22.23±24.70	0.001*
nCD64 MFI†	2011.28±2261.89	502.46±360.81	0.001*
nCD64 Mean FI	2137.19±2319.71	557.30±393.70	0.001*
N:L CD64 index	7.29±9.66	1.85±1.83	0.001*

[Table/Fig-3]: CD64 expression in cases as compared to controls.
Chi-square test was applied; The p-value<0.05 was considered statistically significant; *Statistically significant; †Median Fluorescent intensity; Median Fluorescent Intensity (MFI); Mean Fluorescent intensity (FI)

Parameters	Cut-off	Sensitivity	Specificity	Positive Predictive Value	Negative predictive value
Percentage of neutrophils expressing CD64	56.7%	67.4%	83.7%	80.5%	72%
nCD64 MFI*	682	67.4%	81.4%	78.3%	71.4%
nCD64 mean FI	763	65.1%	81.4%	77.8%	70%
N:L CD64 index	2.53	60.4%	81.4%	76.4%	67.3%

[Table/Fig-4]: Cut offs, sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the four flow cytometric parameters.
*Median fluorescent intensity; Mean Fluorescent Intensity (FI)

Parameters in patients	Percentage of neutrophils expressing CD64		nCD64 MFI		nCD64 Mean FI		N:L CD64 index	
	High (n,%)	Low (n,%)	High (n,%)	Low (n,%)	High (n,%)	Low (n,%)	High (n,%)	Low (n,%)
CRP† abnormal (n=23)	15 (65)	8 (35)	17 (73.9)	6 (26.1)	17 (73.9)	6 (26.1)	7 (30.4)	16 69.6)
CRP normal (n=20)	7 (34.8)	13 (65.2)	5 (25)	15 (75)	7 (35)	13 (65)	1 (5)	19 (95)
p-value	0.04*		0.001*		0.01*		0.03*	
PCT‡ abnormal (n=35)	21 (60.0)	14 (40.0)	21 (60.0)	14 (40.0)	23 (65.7)	12 (34.3)	7 (20.0)	28 (80.0)
PCT normal (n=8)	1 (12.5)	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	7 (87.5)	1 (12.5)	7 (87.5)
p-value	0.02*		0.01*		0.006*		0.6	

[Table/Fig-5]: Association of CRP and PCT with Percentage of neutrophils expressing CD64, nCD64 MFI, nCD64 Mean FI and MFI CD64 neutrophils/MFI CD64 lymphocytes (N=43).
The p-value <0.05 was considered statistically significant; *Statistically significant, †C-Reactive Protein, ‡Procalcitonin; Median Fluorescent Intensity (MFI); Mean Fluorescent Intensity (FI)

Using 90th percentile values of controls (excluding four outliers) for percentage of neutrophils expressing CD64, MFI, mean fluorescent intensity and ratio of MFI CD64 neutrophil and MFI CD64 lymphocyte, the cut offs, sensitivity, specificity, PPV and NPV were calculated [Table/Fig-4].

A statistically significant difference was seen between sepsis cases with abnormal and normal CRP and PCT when compared for percentage of neutrophils expressing CD64, nCD64 MFI and nCD64 Mean FI [Table/Fig-5].

DISCUSSION

Sepsis is a life-threatening condition that can affect any age group with a high consequent mortality. There is no single investigation that can conclusively diagnose sepsis promptly and specifically. Microbial culture is considered the gold standard for diagnosis of sepsis, but is limited by its low sensitivity and higher turnaround time. The provisional culture reports take 24-48 hours to be available while a confirmed negative report is received after 1 week [7]. On the other hand, by flow cytometry, results can be obtained in an hour. Flow cytometry has been utilised recently for evaluating role of expression of CD64, CD11b, CD59, CD45RO, CD10, CD16 and CD25 in diagnosing or prognosticating sepsis cases [7,10].

CD64 or FcγRI is a surface marker for monocytes however, its increased expression on neutrophils is one of the antigenic changes seen during acute inflammatory response. This increased expression of CD64 on neutrophils in sepsis could be attributed to increased levels of cytokines and Granulocyte- Colony Stimulating Factor (G-CSF) in response to microbial pathogens. This receptor is required to mediate monocyte/neutrophil cytotoxicity and activation of respiratory burst and its expression on neutrophils is regulated in a graded pattern depending on extent of inflammatory response to tissue damage [8,11-13].

Paul D et al., Sarode R et al., El-Mazary AAM et al., and Pradhan R et al., have evaluated the role of CD64 in diagnosis of neonatal sepsis while Bae MH et al., Dimoula A et al., and Icardi M et al., studied the same in diagnosis of adult sepsis [7,10,14-18]. These studies revealed that increased neutrophilic CD64 expression is of diagnostic use, both in children and adult sepsis [Table/Fig-6] [7,19].

Bae MH et al., and Paul D et al., documented a male preponderance in their septic study population [10,15]. It has been proposed that since factors which regulate synthesis of globulins are encoded by genes on X chromosome, males are less protected immunologically as compared to the females [10,15]. Patients with age in the range of 21-40 years formed the majority of sepsis cases in the present study and included 51.2% males and 48.8% females.

Increased CD64 expression on neutrophils has been reported in previous studies by evaluating various flow cytometric parameters- MFI, mean fluorescent intensity, percentage of neutrophils expressing CD64 and CD64 index (mean fluorescent intensity nCD64/mean fluorescent intensity beads) using quantibrite beads [15,16,20,21]. Present study evaluated percentage of neutrophils expressing CD64, MFI, mean fluorescent intensity and MFI for nCD64:lymphocyte CD64 ratio. The latter parameter, i.e., N:L CD64 index is simple to calculate and also, it does not require quantibrite beads, hence does not increase the cost of test.

Authors	Parameters	Cut-off	Sensitivity	Specificity	PPV	NPV
Present study, 2021	MFI	682	67.4%	81.4%	78.3%	71.4%
Sarode R et al., 2017 [7]	MFI	37.55	96.77%	100%	-	-
Dimoula A et al., 2014 [18]	MFI	230	89%	87%	-	-
Groselj-Grenc M et al., 2008 [20]	MFI (Day 0 and 1)	72 (day 0) 65 (day 1)	65.5% (day 0) 95.5% (day 1)	92.6% (day 0) 95% (day 1)	-	-
Pradhan R et al.,, 2016 [17]	MFI	126.1	73.01%	89.18%		
Present study, 2021	Neutrophil % age expression	56.7%	67.4%%	83.7%%	80.5%	72%
Paul D et al., 2015 [15]	Neutrophil % age expression	67.73±24.74	100%	40%	25%	100%
Present study, 2021	Mean FI	763	65.1%	81.4%	77.8%	70%
Danikas DD et al.,, 2008 [25]	Mean FI	2.45	60%	100%	100%	53.8%

[Table/Fig-6]: Comparison with previous studies [7,15,17,18,20,25].
Positive Predictive Value (PPV) and Negative Predictive Values (NPV); Median Fluorescent Intensity (MFI); Mean Fluorescent Intensity (FI)

In current study, the sepsis patients showed increased expression of CD64 as compared to controls, in terms of percentage of neutrophils expressing CD64 (63.88±34.12 in cases), mean FI (2137.19±2319.71), MFI (2011.28±2261.89) as well as N:L CD64 index (7.29±9.66) and the results were statistically significant. Zhou Y et al., also found CD64 positive neutrophils percentage and magnitude of CD64 expression by neutrophils to be significantly increased in sepsis patients [22]. A comparison of the sensitivity, specificity, NPV and PPV with other previous studies is shown in [Table/Fig-6] [7,15,17,18,20,23].

Sensitivity and specificity of each parameter evaluated in present study varied from 60.4-67.4% and 81.4-83.7%, respectively. Sensitivity of mean fluorescent intensity of nCD64 was found to be marginally higher (67.4%) than other parameters while specificity of percentage of neutrophils expressing CD64 was found to be highest (83.7%). Icardi M et al., reported CD64 index as effective and inexpensive in predicting infection in the postoperative patients, when followed-up daily for 7 days [14].

In present study, specificity of nCD64 expression by any of the parameters included in the study was reasonably high with a high PPV, but had low sensitivity. This disparity could be attributed to administration of antibiotics prior to blood sampling in all cases in our study and greater time gap between onset of sepsis/hospital admission and collection of blood (>72 hours) for analysis of nCD64 expression. This is in concordance with the findings of Fjaertoft G et al., who found that CD64 expression by neutrophils was significantly increased even after 3 days of administering antibiotics in case of bacterial infections [19]. Most of the other studies have excluded cases who had received antibiotics [15,17]. Moreover, variable sensitivity and specificity of nCD64 expression for diagnosis of sepsis in different studies can be attributed to following non standardised evaluation techniques [24,25]. Factors impacting the results may include patient selection variability, time of blood collection (day 0/1 versus serial determinations), differently labeled antibodies, parameter assayed (MFI/mean fluorescent intensity/ percentage of neutrophils expressing CD64/CD64 index using beads), usage of different clones and fluorochrome conjugations of antibodies [14,24,25].

In current study, most of the samples (81.39%) were collected after 72 hours of admission and all patients had received antibiotics. Previous studies have suggested that nCD64 expression increases within 4 hours of infection and remains stable for atleast 24 to 72 hours, making it a good parameter to diagnose sepsis [7,15]. According to some authors, nCD64 expression in patients with sepsis was higher on day 0 and have reported a significant decrease by day 3 or day 8 [9,18].

When comparing flow cytometric parameters of CD64 expression on neutrophils with normal and abnormal CRP and PCT values in sepsis patients, a statistically significant difference was seen in the present study (except N:L CD64 index). Combining parameters like CRP with nCD64 MFI can increase the sensitivity for sepsis diagnosis [18].

Limitation(s)

Our institution being a referral centre, most of the patients had received antibiotics before collection of blood sample. Also, the time lag between onset of infection/admission in majority of the patients was more than 72 hours in the present study.

CONCLUSION(S)

To conclude, quantitative expression of nCD64, by flow cytometry, in terms of percentage expression, MFI, mean fluorescent intensity and N:L CD64 index is helpful in diagnosing sepsis patients even after 72 hours of onset, especially in conjunction with other laboratory tests like CRP, ANC and PCT. This is a rapid, reliable and cost effective investigation (especially in developing country like India), with a greater specificity than sensitivity in diagnosing sepsis. More studies are required to clearly define role of nCD64 expression after standardising pre-analytical and analytical variables.

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