

## Increased Expression Level of Integrin $\alpha 4$ on Circulating Neutrophils in Septic Pneumonia Cases

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### Abstract

**Background:** Severe pneumonia often complicates sepsis, and is known as a main causes of elderly death. In pneumonia, neutrophils play an important role in acute lung injury and involvement of CD11b/CD18 on neutrophils has been well studied. Although expression level of integrin  $\alpha 4\beta 1$  on neutrophil is also known to be up-regulated by inflammatory cytokines, its involvement of pneumonia and septic state is not fully understood.

**Methods:** The purpose of this study is to address the possible involvement of integrin  $\alpha 4$  on circulating neutrophils and its correlation with clinical findings in septic pneumonia cases. We measured the expression levels of CD11b and integrin  $\alpha 4$  on peripheral neutrophils, serum procalcitonin level, neutrophil counts, CRP, and severity of illness (SOFA score) in subjects with septic pneumonia cases, non-septic pneumonia cases, and control.

**Results:** Both expression levels of CD11b and integrin  $\alpha 4$  were significantly higher in septic cases compared with control. And both of CD11b and  $\alpha 4$  showed weak but significant correlation with SOFA scores. In non-septic pneumonia cases, expression level of CD11b was significantly higher than control cases, but integrin  $\alpha 4$  did not show the statistical difference.

**Conclusions:** These findings suggest that up-regulated integrin  $\alpha 4$  and CD11b on systemic neutrophils might be associated with septic state.

**Keywords:** Integrin  $\alpha 4$ ; CD11b; Sepsis; Pneumonia

### Abbreviations

SOFA: Sequential Organ Failure Assessment; qSOFA: Quick Sequential Organ Function Assessment; CRP: C-Reactive Protein; WBC: White Blood Cell Count; FITC: Fluorescein Isothiocyanate; MFI: Mean Fluorescent Intensity; PBS: Phosphate Buffered Saline; SD: Standard Deviation

### Introduction

Although acute pneumonia is a common disease, the mortality of elderly pneumonia patients still remains high [1]. Severe bacterial pneumonia can easily develop sepsis and acute lung injury. Neutrophils play the central role in acute lung injury of pneumonia, and circulating neutrophils are recruited into the alveolar space by transmigrating across vascular endothelial cells. We previously reported the involvement of neutrophil elastase in acute lung injury in animal model [2]. Integrins are heterodimeric, transmembrane proteins, and are thought to be involved in the maintenance of cell adhesion and tissue integrity. The complexity and diversity of different integrins are

implicated in different lung diseases [3]. Integrin  $\alpha M\beta 2$  (CD11b/CD18) plays an important role in extravasation from the circulating blood flow into the site of alveolar inflammation [4], but CD18-independent neutrophil transmigration pathways have also been reported [5]. Although the adhesion molecules that mediate CD18-independent pathways still remained uncertain, integrin  $\alpha$  subfamily is one of the candidates [6]. Some of integrin  $\alpha$  subfamily ( $\alpha 2\beta 1, \alpha 3\beta 1, \alpha 4\beta 1, \alpha 5\beta 1, \alpha 6\beta 1$  and  $\alpha 9\beta 1$ ) are known to be expressed on peripheral neutrophils, and are up-regulated upon activation [7]. We recently reported up-regulated integrin  $\alpha 9\beta 1$  on systemic neutrophils and related increasing serum IL-17A in elderly patients with aspiration pneumonia [8]. Integrin  $\alpha 4\beta 1$  (CD49d/CD29) is constitutively expressed on the cell surface of lymphocytes, monocytes, neutrophils, and eosinophils, and is known to be up-regulated by stimuli including inflammatory cytokines [9]. The  $\alpha 4$  subunit cDNA sequence is more than 40% identical to the integrin  $\alpha 9$  subunit sequence, but less than 30% identical to any other integrin  $\alpha$  subunit, showing  $\alpha 4$  and  $\alpha 9$  as the specific members of integrin  $\alpha$  subfamily [10]. Therefore, we speculated that peripheral integrin  $\alpha 4$  on neutrophils may work like integrin  $\alpha 9$  and hypothesized that integrin  $\alpha 4$  on peripheral neutrophils might be up-regulated and mediated neutrophilic lung injury in septic pneumonia cases.

## Material and Methods

**Study design:** The study was carried out in Akiota Hospital (Hiroshima, Japan) from April 1, 2010 to March 31, 2013. The subjects were as following; septic pneumonia cases ( $n = 18$ ; 10 males and 8 females,  $66.2 \pm 7.4$  years old), non-septic pneumonia cases ( $n = 15$ ; 8 males and 7 females,  $68.6 \pm 7.2$  years old), and healthy subjects as the control group ( $n = 10$ ; 6 males and 4 females,  $67.1 \pm 4.2$  years old). They were recruited from our hospital. All subjects were given informed consent for participation in the study, which was approved by local ethics committees of Akiota Hospital. At the point of diagnosis of pneumonia, serum C-reactive protein (CRP), white blood cell count (WBC), serum procalcitonin concentration, and expression levels of CD11b and integrin  $\alpha 4$  (CD49d) on peripheral neutrophils were measured. The severity of pneumonia was graded by CURB-65 score, which was counted (range 0 - 5 points; with or without altered mental status, uremia, increased respiratory rate, low blood pressure, and older age) in each subject [11]. Sepsis was defined by showing positive blood culture with systemic illness. Sequential organ failure assessment (SOFA) scores were calculated as the severity of illness at the same day for each case as previously described [12,13]. Criteria for a systemic ill consisted of 2 of the manifestations of sepsis as following; altered mental status, tachypnea (respiratory rate  $> 22$ /minute), hypotension (systolic blood pressure  $< 100$  mmHg) (quick sequential organ function assessment (qSOFA)) [11].

**Isolation of circulating neutrophils:** On admission day, neutrophils were isolated from peripheral venous blood containing heparin, and were isolated by Ficoll-Hypaque density gradient centrifugation, followed by 3% dextran sedimentation as our previously reported [14]. Red blood cells were treated with hypotonic lysis, and then neutrophils were washed and re-suspended in phosphate buffered saline (PBS).

**Reagents and antibodies:** Fluorescein isothiocyanate (FITC) - conjugated mouse monoclonal antibody HP2/1 (IgG1, anti-human integrin  $\alpha 4$ ), FITC - conjugated mouse monoclonal antibody Bear1 (mouse IgG1, anti-human CD11b), and isotype-matched control antibody were purchased from BD Pharmingen (San Diego, CA).

**Flow cytometric analysis:** Integrin expression level on neutrophil was analyzed as previously reported [15]. Isolated neutrophils suspended with 100  $\mu$ l of PBS were incubated with FITC - conjugated antibodies (anti - integrin  $\alpha 4$ , anti - CD11b, or control antibody) for 20 minutes at 4°C. Then neutrophil cell pellets were washed with PBS to remove non - binding antibody, and the cell pellets were re-suspended in 100  $\mu$ l of PBS. The level of fluorescence on 10,000 neutrophils was measured by flow cytometry (Becton Dickinson, Mountain View, CA). The relative mean fluorescent intensity (MFI) was calculated as following; MFI level of neutrophils treated with anti - integrin antibody divided by MFI level of the non-specific isotype-matched control antibody.

**Statistical Analysis:** Each data was expressed as mean  $\pm$  standard deviation (SD) of the mean. Statistical analysis was performed by computer software (Excel Statistics 2012, SSRI Co., Ltd., (Tokyo, Japan) and KaleidaGraph 4.1, Synergy Software Corp., (Reading, PA)). A one-way analysis of variance followed by Fisher's least significant difference test was used to detect differences among groups, and Pearson correlation coefficient was used to analyze the relationship between the groups. Probability value of less than 0.05 was considered as statistically significant.

**Result**

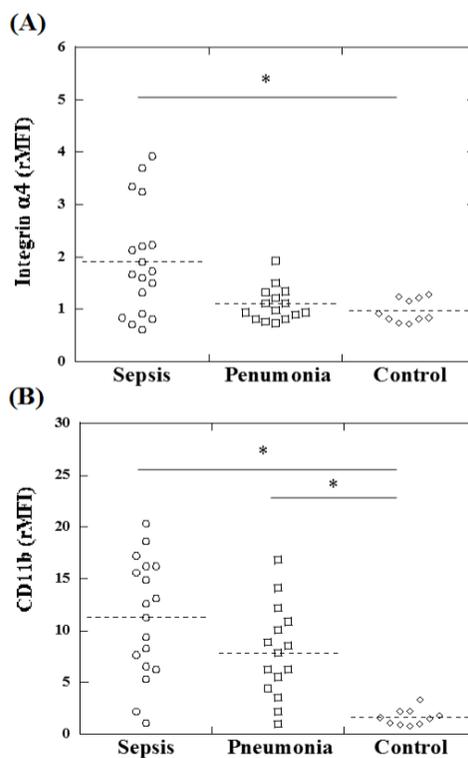
The clinical characteristics of the subjects are shown in table 1. In sepsis group, WBC, neutrophil count, CRP, procalcitonin, CURB-65 point, and SOFA score were significant higher than those of control group (each p value were 0.0006, 0.0177, < 0.0001, < 0.0001, < 0.0001 and < 0.0001). And in non-septic pneumonia group, WBC, neutrophil count, CRP, procalcitonin, and CURB-65 point were also higher than those of the control (each p value were 0.0121, 0.0197, 0.0026, 0.0371, and 0.0142), but there was not significant difference about SOFA score (p = 0.4823). And when comparing between septic pneumonia group and non-septic pneumonia group, procalcitonin, CURB-65 point, and SOFA score were significant higher in sepsis group (each p value were 0.0087, 0.0129 and < 0.0001).

	Sepsis	Pneumonia	Control	p value
Number	18	15	10	
Age	66.2 ± 7.4	68.6 ± 7.2	67.1 ± 4.2	0.4916
Gender (M/F)	10; 8	8; 7	6; 4	0.5135
WBC (mm <sup>3</sup> )	12,120.0 ± 4754.5	10,632.0 ± 3,155.8	5,712.0 ± 1,160.0	0.0005
Neutrophil count (mm <sup>3</sup> )	8,054.4 ± 4,637.3	7,706.0 ± 2,907.1	3,463.0 ± 1,189.5	0.0074
CRP (mg/dL)	11.89 ± 5.54	8.02 ± 3.99	0.25 ± 0.13	< 0.0001
Procalcitonin (ng/mL)	0.67 ± 0.40	0.24 ± 0.12	0.04 ± 0.03	< 0.0001
CURB-65 (point)	3.5 ± 0.9	2.0 ± 0.7	0.0 ± 0.0	< 0.0001
SOFA score (Point)	3.3 ± 0.6	0.5 ± 0.6	0.00 ± 0.00	< 0.0001
				(Mean ± SD)

**Table 1:** Clinical characteristics of subjects with sepsis, pneumonia, and control.

All data is expressed as means ± standard deviation of the means. Each p value showed the difference among the 3 groups. Sequential organ failure assessment (SOFA), C-reactive protein (CRP), white blood cell count (WBC).

The expression levels of integrin  $\alpha 4$  and CD11b among the groups are shown in figure 1. The expression levels of integrin  $\alpha 4$  and CD11b at the point of admission of the sepsis cases were significantly higher than that of the control (each p value were 0.0286 and 0.0001), and CD11b of pneumonia cases was also higher compared with the control (p value was 0.0096). In non-septic pneumonia group, level of integrin  $\alpha 4$  were not significant different from the control group (p = 0.8748).



**Figure 1:** The expression levels of integrin  $\alpha 4$  and CD11b in sepsis, pneumonia, and control. The levels of integrin  $\alpha 4$  (A), CD11b (B) on neutrophils of three groups (septic group, pneumonia group, and control group) are expressed as relative mean fluorescent intensity (rMFI) (monoclonal antibody/corresponding isotype control antibody). Each bar expressed mean of the group. \*probability value of less than 0.05 compared with the control group.

Next, we compared the correlation of the markers between integrin  $\alpha 4$  and CD11b (Table 2). Expression levels of CD11b and  $\alpha 4$  did not correlate with pneumonia severity score (CURB-65), but SOFA score positively correlated with integrin  $\alpha 4$  or CD11b (p value were 0.0029 and 0.0196). CD11b expression levels did not correlate with those of integrin  $\alpha 4$  ( $r = 0.2376, p = 0.1249$ ). Both of integrin  $\alpha 4$  and CD11b did not show any significant difference when comparing with WBC, neutrophil count, CRP, and serum procalcitonin concentration.

		Integrin $\alpha 4$ (n = 43)	CD11b (n = 43)
WBC (mm <sup>3</sup> )	r-value	0.1608	0.4158
	p-value	0.3029	0.0017
Neutrophil count (mm <sup>3</sup> )	r-value	0.1595	0.2451
	p-value	0.307	0.1132
CRP (mg/dL)	r-value	0.281	0.2358
	p-value	0.0679	0.1485
Procalcitonin (ng/mL)	r-value	0.2702	0.2553
	p-value	0.0797	0.1229
CURB-65 (point)	r-value	0.2929	0.252
	p-value	0.2219	0.1217
SOFA score (Point)	r-value	0.4431	0.3822
	p-value	0.0029	0.0196
Integrin $\alpha 4$ (rMFI)	r-value	-	0.3822
	p-value	-	0.0196
CD11b (rMFI)	r-value	0.2376	-
	p-value	0.1249	-

**Table 2:** Correlation between integrin  $\alpha 4$  and CD11b in sepsis, pneumonia, and control.

Pearson correlation coefficient was used to analyze the relationship between the makers in all subjects (sepsis group, pneumonia group, and control group). Sequential organ failure assessment (SOFA), C-reactive protein (CRP), white blood cell count (WBC), relative mean fluorescent intensity (rMFI).

## Discussion

In the present study, we demonstrated the increased expression levels of integrin  $\alpha 4$  and CD11b on circulating neutrophils in septic pneumonia cases, and increased expression levels of CD11b in non-septic pneumonia cases. But there was no statistically significant correlation between pneumonia severity (point counts of CURB-65) and expression level of integrin  $\alpha 4$  and/or CD11b. So far, several previous reports showed increased expression of integrin  $\alpha 4$  on septic peripheral neutrophils [16,17]. As long as we know, this is the first report showing comparison of integrin  $\alpha 4$  level between septic pneumonia patients and non-septic pneumonia patients. Previous reports were analysis about septic cases including non-pneumonia cases, and their causes of sepsis were heterogeneous. Therefore, effect upon septic pneumonia could be directory compared in our study. In 2016, the criteria of sepsis has been changed, and SOFA score is recommended for estimating the severity of illness [12,13]. We also compared the relation among CD11b, integrin  $\alpha 4$  and SOFA score, and show that there was weak but significant positive correlation. So far, there are no reports comparing SOFA score and integrin  $\alpha 4$  expression levels like us, before.

In this study, expression levels of integrin  $\alpha 4$  and CD11b were positively correlated with SOFA score, but CD11b expression levels did not correlate with those of integrin  $\alpha 4$ . This might suggest the possible existence of a CD11b/CD18-independent neutrophil migration mechanism of integrin  $\alpha$  subfamily including integrin  $\alpha 4\beta 1$ . Previously, possibility of cross-talking signaling pathways playing a role in modifying migration between CD11b/CD18 and  $\beta 1$  integrin has been also reported [18], but the precise mechanism of the activation of integrin  $\alpha 4\beta 1$  and CD11b/CD18 relationship in sepsis is still remained unclear. For example, it might be possible to predict the risk of sepsis by measuring increased integrin  $\alpha 4$  expression level before showing the results of bacterial culture in the future. But from the result of this study, we could not conclude such like above description. Therefore, further investigation will be necessary.

The difference between our analysis and previous reports was that a few studies [19,20] showed decreased expression of integrin  $\alpha 4$  and CD11b in septic cases. They reported as the reason that lower expression on neutrophils could be a result of receptor internalization. The reason of discrepancy was uncertain, but our cases might not meet with such like them. In our analysis, severity of sepsis (SOFA score) was relatively mild compared with previous reports. It was possible that background and complicated disease of the subjects might affect the integrin expression level.

One limitation of our study is that the results are based on circulating neutrophils, not pulmonary leukocytes. Therefore, in this regard, we have not shown direct evidence that integrin  $\alpha 4$  or CD11b is involved in increased neutrophil transmigration from circulating vessels into the alveolar space, the site of inflammation. Another limitation is the number of the sample was rather small, and age of the patient was relative older. We have no data about younger age. In these meaning, further examination would be necessary to figure out the involvement of integrin  $\alpha 4$  and neutrophil migration in detail. And in strict meaning, the control group (healthy subjects) in this study might not matched with age and gender. But there was no significant difference with age and gender among the 3 groups. Although there are some limitations for interpretation, measuring integrin  $\alpha 4$  expression level on neutrophils might be a candidate for simple screening of septic pneumonia cases.

### Conclusion

In summary, the present study suggests that CD11b and integrin  $\alpha 4$  expression level on peripheral neutrophils might be associated with septic pneumonia state.

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None.

### Author Contributions

All authors helped to select the blood samples from study subjects. YT and GT helped to solve experimental problems. YT, YI and YH helped to obtain the clinical data from all subjects. YT and GT conceived the study, participated in its design, and contributed to the manuscript. All authors have read and approved the final manuscript.

### Conflicts of Interest

The authors declare that they have no competing interests.

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