Increased Expression Levels of Integrin $\alpha 9\beta 1$ and CD11b on Circulating Neutrophils and Elevated Serum IL-17A in Elderly Aspiration Pneumonia

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Key Words

Integrin $\alpha 9\beta 1$ · CD11b · IL-17A · Aspiration pneumonia · Elderly

Abstract

Background: Repeated aspiration pneumonia is a serious problem in the elderly. In aspiration pneumonia, neutrophils play an important role in acute lung injury, while CD18-independent neutrophil transmigration pathways have also been reported in acid-aspiration pneumonia animal models. However, the involvement of IL-17A and $\beta 1$ integrin still remains unclear. The $\beta 1$ integrin subfamily integrin $\alpha 9\beta 1$ has been shown to be expressed on human neutrophils and to mediate adhesion to extracellular matrix proteins including the vascular cell adhesion molecule-1. **Objectives:** To elucidate the possible involvement of $\beta 1$ integrin subfamily and IL-17A in aspiration pneumonia. **Methods:** We analyzed the expression levels of CD11b, CD18 and integrin $\alpha 9\beta 1$ in circulating neutrophils and serum concentration of IL-17A, IL-22 and IL-23 in elderly aspiration pneumonia patients ($n = 32$, 14 males and 18 females, 78.8 ± 3.9 years old) at 2 time points (on the day of admission before starting antibiotics and the day after finishing antibiotics) and compared the results with those of a control group ($n = 30$, 13 males and 17 females, 76.1 ± 3.4 years old). **Results:** Recombinant IL-17A stimulated integrin $\alpha 9\beta 1$ and CD11b expression levels in healthy human

neutrophils in vitro. The expression levels of integrin $\alpha 9\beta 1$ and CD11b in circulating neutrophils were significantly higher in pneumonia patients compared with the controls. In addition, serum IL-17A concentration was significantly increased in pneumonia patients. Integrin $\alpha 9\beta 1$ levels positively correlated with serum IL-17A and CD18 expression levels. **Conclusions:** These findings suggest a potential role of integrin $\alpha 9\beta 1$ expressed in neutrophils and elevated serum IL-17A in extravasation of neutrophils in cases of aspiration pneumonia.

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Introduction

Aspiration pneumonia is a heterogeneous disease entity and repeated microscopic aspiration pneumonia is known to be common among elderly suffering from cerebral stroke and dementia [1]. Kikuchi et al. [2] reported that silent aspiration occurred more frequently in Japanese elderly. However, the molecular mechanism of aspiration pneumonia has not been fully elucidated. Neutrophils play the central role in acute lung injury and circulating neutrophils are recruited into the alveolar space by transmigrating across vascular endothelial cells. We previously reported the involvement of neutrophil elastase and superoxide in acute lung injury in a mouse model [3,

4]. As previously reported [5], $\beta 2$ integrin, CD11b/CD18 plays an important role in extravasation from the circulating blood flow into the site of alveolar inflammation, but CD18-independent neutrophil transmigration pathways have also been reported in some animal models, where neutrophil emigration is induced by *Escherichia coli*, *Streptococcus pneumoniae* and hydrochloric acid [6–8]. Although the adhesion molecules that mediate the CD18-independent pathways still remain uncertain, the $\beta 1$ integrin subfamily is one of the candidates [9]. To our knowledge, several members of the $\beta 1$ integrin subfamily ($\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$ and $\alpha 9\beta 1$) are known to be expressed on neutrophils and upregulated upon activation [10]. Tasaka et al. [11] investigated the involvement of integrin $\alpha 4\beta 1$ in CD18-independent neutrophil migration. The $\alpha 9$ subunit cDNA sequence is 41% identical to the integrin $\alpha 4$ subunit sequence, but $\leq 27\%$ identical to any other integrin subunit, putting $\alpha 9$ and $\alpha 4$ as sole members of a subfamily of integrin α subunits [12, 13]. As we previously reported [13], interaction between integrin $\alpha 9\beta 1$ expressed on human neutrophils and vascular cell adhesion molecule (VCAM)-1 on the surface of vascular endothelial cells plays an important role in extravasation of neutrophils to the site of inflammation. Glynn et al. [14] reported upregulated CD11b expression on systemic neutrophils in community-acquired pneumonia (CAP) patients, suggesting systemic neutrophils are primed in these patients. Therefore, we hypothesized that $\alpha 9\beta 1$ integrin might be increased on systemic neutrophils and the expression level might be upregulated independently of CD11b/CD18 expression.

On the other hand, the T helper 17 cell-produced cytokine IL-17A has been implicated in the development of neutrophilic airway inflammation and IL-17A-related neutrophilic accumulation in COPD and viral pneumonia [15, 16]. However, the involvement in aspiration pneumonia still remains uncertain. We hypothesized that IL-17A and members of the $\beta 1$ integrin subfamily might associate in human neutrophilic lung injury. To elucidate the role of integrin $\alpha 9\beta 1$ in neutrophil emigration during bacterial pneumonia, we measured serum IL-17A and the expression levels of $\alpha 9\beta 1$, CD11b and CD18 in circulating neutrophils in elderly aspiration pneumonia.

Materials and Methods

Study Design

The pneumonia group consisted of 32 individuals (14 males and 18 females, 78.8 ± 3.9 years old), recruited from Shimane University Hospital (Izumo, Japan), Aki-Ohta Hospital (Hiroshi-

ma, Japan) and Mazda co-operation Hospital (Hiroshima, Japan). All patients were more than 70 years old. They comprised 14 CAP patients and 18 nursing and healthcare-associated pneumonia (NHCAP) patients. The Japan Respiratory Society Guidelines documented this new category [17], which describes patients with any of the following: residence in a long-term nursing home setting or healthcare home; discharge from hospital during the preceding 90 days; elderly persons or physically disabled persons who need healthcare, or persons continuously receiving endovascular therapy in an ambulatory setting. Thirty non-pneumonia individuals without respiratory disease, sex- and age-matched, were recruited from the same hospitals to act as the control group for this study (13 males and 17 females, 76.1 ± 3.4 years old). At the point of diagnosis, serum C-reactive protein and white blood cell count (WBC) were measured. The severity of pneumonia was graded by CURB-65 score (confusion, uremia, respiratory rate, low blood pressure, age 65 years or greater) [18]. Count of points by PORT-study was also used as a pneumonia severity index [19]. Aspiration pneumonia was graded as mild, CURB-65 score of 1 ($n = 13$), moderate, CURB-65 score of 2 ($n = 16$) or severe, CURB-65 score of 3 ($n = 3$). The diagnosis of aspiration pneumonia was performed by identification of an infiltrative shadow on a chest X-ray, increased peripheral WBC and underlying aspiration disorder. Clinical signs suggesting the presence of dysphasia included delayed swallowing and coughing or choking before, during and after swallowing [20]. To confirm the presence of dysphasia in the patient group without an episode of obvious aspiration, a water-swallowing test, videofluoroscopic swallow assessment or swallowing provocation test was performed after improvement as previously reported [21]. Two patients diagnosed as having aspiration pneumonia occurring after an episode of apparent vomiting who were graded with severe pneumonia (CURB-65 score of 3) died of acute respiratory failure, while the remainder all improved. All subjects gave written informed consent for participation in the study, which was approved by the institutional review board of Shimane University School of Medicine (approval number: 360-20-5-14), and local ethics committees of Aki-Ohta Hospital and Mazda co-operation Hospital.

Serum and Neutrophil Isolation

Venous blood samples were drawn from the patients at 2 time points: on the day of admission before starting antibiotics and on the day after finishing antibiotics. Serum was rapidly centrifuged, frozen at -80°C and stored until measurement. Neutrophils were purified from human peripheral venous blood containing 20 U/ml of heparin and were isolated by Ficoll-Hypaque density gradient centrifugation, followed by 3% dextran sedimentation as we previously reported [13]. Erythrocytes were subjected to hypotonic lysis and remaining neutrophils were washed and resuspended in phosphate-buffered saline (PBS).

Reagents, Antibodies, Cells and Cell Culture

Recombinant human tumor necrosis factor (TNF)- α was obtained from R&D Systems (San Diego, Calif., USA). Recombinant human IL-17A was obtained from Pepro Tech EC (London, UK). Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibody Bear1 (mouse IgG1, anti-human CD11b) and FITC-conjugated monoclonal antibody 7E4 (mouse IgG1 anti-human CD18) were purchased from BD Pharmingen (San Diego, Calif., USA). Purified mouse monoclonal antibody Y9A2 (mouse IgG1

Fig. 1. Increased expression levels of integrin $\alpha 9\beta 1$ (a) and CD11b (b) on neutrophils after being stimulated with IL-17A. Bars show MFI of integrin $\alpha 9\beta 1$ and CD11b on healthy human neutrophils after being stimulated with various concentrations of IL-17A (0, 0.1, 1.0, 10.0, 100.0 ng/ml). Each group consisted of 4 samples and the data is expressed as means $\pm$ SD. * $p < 0.05$ compared with non-stimulation (0 ng/ml). c IL-17A stimulating integrin $\alpha 9\beta 1$ and CD11b mediating neutrophils transmigration through the activated HUVE cells monolayer. Purified healthy human neutrophils that had been incubated with no antibody or antibody to $\alpha 9\beta 1$ (Y9A2), CD18 (IB4), a combination of $\alpha 9\beta 1$ and CD18, or control antibody (R6G9) were added to the top chambers above microporous chambers containing monolayer of HUVE cells that had been incubated with 3 ng/ml of TNF- α . After incubation, neutrophils which migrated across the monolayer with or without serum-free medium with 1.0 ng/ml of IL-17A were collected from the bottom chamber and counted. Each group consisted of 4 samples and the data is expressed as means $\pm$ SD. * $p < 0.05$ compared with the no antibody group.

anti-human integrin $\alpha 9\beta 1$) was purchased from Chemicon International (Temecula, Calif., USA) and labeled using a FITC-labeling kit (Kirkegaard & Perry Laboratories, Gaithersburg, Md., USA) for flow cytometry. For neutrophil transmigration assays, the functional blocking monoclonal antibody IB4 (mouse IgG2a, anti-human CD18) and control antibody R6G9 (mouse IgG2a, anti-human integrin $\beta 6$) were purchased from Merck Millipore (Schwalbach, Germany). Human umbilical vein endothelial (HUVE) cells were purchased from Clonetics (Walkersville, Md., USA) and grown in endothelial cell growth media (Clonetics) and used between passage 3 and 10.

Enzyme-Linked Immunosorbent Assay

Serum IL-17A, IL-22 and IL-23 were measured by human ELISA Kit (IL-17A from Invitrogen Life Technologies, Carlsbad, Calif., USA; IL-22 and IL-23 from R&D Systems) according to the manufacturers' instructions. The minimum detectable values were 2.0 pg/ml for IL-17A, 2.7 pg/ml for IL-22 and 6.8 pg/ml for IL-23. All samples were assayed in duplicate.

Flow Cytometry

Cell surface expression was measured as we previously reported [13]. Briefly, isolated neutrophils were rinsed with PBS. Non-specific binding was blocked with normal goat serum at 4°C for 10 min. Cells were then incubated with antibodies (FITC-conjugated

anti-CD11b, anti-CD18 and anti- $\alpha 9\beta 1$ antibody) for 20 min at 4°C. After that the cell pellets were washed with PBS twice by centrifuging, then the stained cells were re-suspended in 100 μ l of PBS and fluorescence was quantified on 10,000 cells with a FACSscan (Becton Dickinson, Mountain View, Calif., USA). The relative mean fluorescent intensity (MFI) was calculated as the MFI of neutrophils treated with specific antibodies divided by the MFI of the non-specific isotype-matched control antibody. All experiments were carried out in the presence of an isotype control antibody.

Neutrophil Transmigration Assays

Transendothelial neutrophil migration was assessed as previously reported [22]. HUVE cells were plated onto polycarbonate inserts (Transwell, 6.5-mm diameter, 8- μ m pore for 24-well plate; Corning, Lowell, Mass., USA) in 200 μ l of serum-containing endothelial cell growth media and allowed to grow to confluence. Twenty-four hours before addition of neutrophils, the upper chambers were washed twice with serum-free media and new medium was added with 3 ng/ml of TNF- α . Immediately before the addition of neutrophils, the upper chambers were washed twice and medium in the lower chamber was replaced with serum-free DMEM with 1 ng/ml of IL-17A. Purified neutrophils were incubated with anti- $\alpha 9\beta 1$ antibody (10 μ g/ml), anti-CD18 antibody (10 μ g/ml) and anti- $\beta 6$ integrin antibody as the control antibody (10 μ g/ml) for 20 min at 4°C, and 2×10^5 cells in 200

Table 1. Clinical characteristics of aspiration pneumonia patients (n = 32)

Age, years	78.8 ± 3.9
Gender (male:female)	14:18
CRP, mg/dl	11.21 ± 6.83
WBC, /μl	10,892.8 ± 4,157.7
CURB-65 score count	
0	0 (0.0)
1	13 (40.6)
2	16 (50.0)
3	3 (9.4)
4	0 (0.0)
5	0 (0.0)
Average score	1.69 ± 0.64
PORT-study PSI count of points	
0–70	1 (3.1)
71–90	9 (28.1)
91–130	19 (59.4)
131–	3 (9.4)
Average points	92.84 ± 16.88

Data represent number of patients (%) or mean ± SD. Severity of pneumonia graded by CURB-65 score [18] and PORT-study points [19]. CRP = C-reactive protein; PSI = pneumonia severity index.

μl of media were added to each upper chamber. After 3 h at 37°C in 5% CO₂, non-adherent cells in the upper chamber were removed. The medium, including migrated neutrophils from the lower chamber, was collected. Then the lower chamber was rinsed several times to collect all the neutrophils that had transmigrated. The medium and all washes were pooled and re-suspended, and cells were counted with a hemocytometer. All determinations were carried out in duplicate and repeated twice. Each group consisted of 4 samples and the transmigration (%) was expressed as mean ± SD.

Statistical Analysis

Statistical analysis was performed using computer software (KaleidaGraph 4.1; Synergy Software Corp., Reading, Pa., USA). All data were expressed as mean ± SD. Pearson correlation coefficient was used to analyze the relationship between the markers. One-way analysis of variance followed by Fisher's least significant difference test was used to detect differences among groups, and $p < 0.05$ was considered statistically significant.

Results

To clarify whether recombinant IL-17A affects integrin $\alpha 9\beta 1$ expression in healthy human neutrophils, we examined $\alpha 9\beta 1$ and CD11b expression levels in peripheral neutrophils. As shown in (fig. 1a, b), integrin $\alpha 9\beta 1$ and CD11b expression levels were upregulated after stimulation by re-

combinant IL-17A in a concentration-dependent manner (0, 0.1, 1.0, 10.0 and 100.0 ng/ml). Integrin $\alpha 9\beta 1$ expression levels stimulated by recombinant IL-17A (100.0 ng/ml) were significantly higher than that without stimulation (0 ng/ml; $p = 0.0306$), and CD11b expression levels stimulated by recombinant IL-17A (10.0 and 100.0 ng/ml) were significantly higher than that without stimulation (0 ng/ml; $p = 0.0298$ for 10.0 ng/ml; $p = 0.0034$ for 100.0 ng/ml).

Next, we examined whether human neutrophils stimulated with recombinant IL-17A transmigrated through activated HUVE monolayers or not. When neutrophils were stimulated with 1 ng/ml of IL-17A, 25.7% of healthy human neutrophils transmigrated through TNF- α -activated HUVE cells, while the control antibody did not inhibit the migration at all (fig. 1c). Anti-integrin $\alpha 9\beta 1$ antibody partially (17.5%) inhibited neutrophil transmigration ($p = 0.0273$), as did anti-CD18 antibody (15.6%), albeit not significantly. The combination of anti-integrin $\alpha 9\beta 1$ and anti-CD18 antibody more intensely (27.6%) inhibited neutrophil migration than either alone ($p = 0.0167$).

Next, we examined whether or not the integrin expression of circulating neutrophils in pneumonia patients also showed an increase. Thirty-two hospitalized patients were included in the study between January 1, 2008 and September 30, 2010. Clinical characteristics of the patients are shown in (table 1).

The expression levels of integrin $\alpha 9\beta 1$, CD18 and CD11b in aspiration pneumonia patients and controls are shown in table 2 and figure 2. The expression levels of integrin $\alpha 9\beta 1$ and CD11b at the point of admission of the pneumonia patients were significantly higher than those of the controls ($p = 0.0091$ for integrin $\alpha 9\beta 1$; $p = 0.0087$ for CD11b), and integrin $\alpha 9\beta 1$ at the point of admission was higher compared with that after recovery ($p = 0.0128$). There was no significant difference in CD18 expression levels among the 3 groups ($p = 0.1322$).

Serum IL-17A concentration was significantly elevated in pneumonia patients at the point of admission compared with the recovery point and with the control group ($p = 0.0261$ for recovery point; $p = 0.0056$ for control group; table 2, fig. 3). Serum IL-22 concentration was also higher in pneumonia patients compared with the control, but there was no significant difference among the groups ($p = 0.3562$; table 2, fig. 3). Serum IL-23 was undetectable in patients and the control group (table 2).

Next, we compared the correlation among IL-17A, integrin $\alpha 9\beta 1$, CD18 and CD11b (table 3). All subjects

Fig. 2. The expression levels of integrin $\alpha 9\beta 1$, CD18 and CD11b in aspiration pneumonia patients and controls. The levels of integrin $\alpha 9\beta 1$ (a), CD11b (b) and CD18 (c) on neutrophils of three groups (pneumonia group on admission, pneumonia group on recovery and control group) are expressed as relative MFI (monoclonal antibody/corresponding isotype control antibody). All data expressed as mean $\pm$ SD. Data for 2 patients from the group on recovery are absent due to death from pneumonia. * $p < 0.05$ compared with the control group.

(pneumonia group on admission, pneumonia group on recovery and control group) were used for analysis. IL-17A levels positively correlated with integrin $\alpha 9\beta 1$ expression levels ($r = 0.47$, $p = 0.0033$). Integrin $\alpha 9\beta 1$ expression levels positively correlated with CD18 expression levels ($r = 0.28$, $p = 0.0065$). CD18 expression levels also correlated with CD11b ($r = 0.21$, $p = 0.0467$).

Discussion

In the present study we demonstrated the presence of increased expression levels of integrin $\alpha 9\beta 1$ and CD11b on circulating neutrophils and elevated serum IL-17A concentrations in elderly aspiration pneumonia patients. Furthermore, $\alpha 9\beta 1$ statistically correlated with both serum IL-17A concentrations and CD18 expression levels.

Fig. 3. IL-17A and IL-22 serum levels in aspiration pneumonia patients and control. The levels of IL-17A (**a**) and IL-22 (**b**) in serum of three groups (pneumonia group on admission, pneumonia group on recovery and control group) are expressed as pg/ml. All data expressed as mean $\pm$ SD. Serum IL-17A and IL-22 were measured using ELISA. The minimum detectable values for IL-17A

were 2.0 and 2.7 pg/ml for IL-22, and the values below minimum detectable levels were recorded as 0 pg/ml. All samples were assayed in duplicate. Data for 2 patients from the group on recovery are absent due to death from pneumonia. * $p < 0.05$ compared with the control group.

Table 2. The expression levels of integrin $\alpha 9\beta 1$ and CD11b on circulating neutrophils and serum IL-17A concentration in aspiration pneumonia patients and controls

	Pneumonia		Control (n = 30)
	admission (n = 32)	recovery (n = 30)	
IL-17A, pg/ml	4.41 $\pm$ 1.62	3.10 $\pm$ 1.47	2.00 $\pm$ 1.39
IL-22, pg/ml	14.07 $\pm$ 7.56	12.68 $\pm$ 7.47	11.32 $\pm$ 7.55
IL-23, pg/ml	ND	ND	ND
Integrin $\alpha 9\beta 1$	76.84 $\pm$ 22.09	55.60 $\pm$ 14.50	50.70 $\pm$ 12.45
CD11b	96.63 $\pm$ 27.68	85.83 $\pm$ 27.17	76.67 $\pm$ 18.45
CD18	112.81 $\pm$ 16.12	104.57 $\pm$ 18.97	101.97 $\pm$ 12.97

The levels of integrin $\alpha 9\beta 1$, CD11b and CD18 in neutrophils of three groups (pneumonia group on admission, pneumonia group on recovery and control group) are expressed as relative MFI (monoclonal antibody/corresponding isotype control antibody). Serum IL-17A, IL-22 and IL-23 were measured using ELISA. All data expressed as mean $\pm$ SD. Data for 2 patients from the group on recovery are absent due to death from pneumonia. ND = Not detectable.

As far as we know, this is the first report showing increased expression of a $\beta 1$ integrin subfamily molecule on peripheral leukocytes of aspiration pneumonia patients, as well as $\beta 2$ integrin correlation.

As shown in figure 1, the inhibitory ratio after adding anti-CD18 antibody was not significant and while a combination of anti-CD18 antibody and anti-integrin $\alpha 9\beta 1$ antibody increased inhibition, it was still not fully inhibited. This suggests the possible existence of a CD18-inde-

pendent mechanism and molecules other than integrin $\alpha 9\beta 1$ that might mediate neutrophil extravasation. The possibility of cross-talking signaling pathways playing a role in modifying migration between CD11b/CD18 and $\beta 1$ integrin has previously been reported [23], but the signal pathway of integrin $\alpha 9\beta 1$ is not well understood. Recently, two papers reported that integrin $\alpha 9\beta 1$ on human neutrophils depends on the activated mitogen-activated protein kinase (MAPK) [24] or phosphoinositol 3-kinase [25] pathway and potentially reduces neutrophil spontaneous apoptosis. Contrary to our hypothesis, our results of flow cytometric analysis showed a statistically significant correlation between CD11b and integrin $\alpha 9\beta 1$, and suggest the possibility of integrin $\alpha 9\beta 1$ sharing the same signal pathway with CD11b/CD18 on neutrophils and playing a certain role in the migration of human neutrophils. Since the precise mechanism of the activation of integrin $\alpha 9\beta 1$ and the $\beta 2$ integrin relationship still remains unclear, further investigation will be necessary.

There was no statistically significant relationship among disease activity parameters (serum C-reactive protein, WBC and point counts of CURB-65 classification, PORT-study pneumonia severity index) and expression levels of integrin $\alpha 9\beta 1$ or CD11b in circulating neutrophils or serum IL-17A concentrations of the pneumonia patients (data not shown). However, $\alpha 9\beta 1$ expression levels showed a significant difference between the point of admission and that of recovery, and most of the aspiration pneumonia patients in this study actually improved. This gives us indirect evidence that expression of these molecules has some relationship with the degree of pneumonia severity. The shortage of patients at the severe end of the spectrum of the infection in our study could be a factor. This may partially account for the weak correlation between these molecule expression levels and pneumonia severity. A previous study reported an increase in circulating neutrophil CD11b expression in acid-aspiration animal models [26] and septic patients [27], which might play a role in organ injury. Hence, we speculate that increased expression levels of integrin $\alpha 9\beta 1$ or CD11b in circulating neutrophils, or increased serum IL-17A concentrations, may have some clinical relevance in aspiration pneumonia patients.

One limitation of our study is that the results are based on peripheral neutrophils, not pulmonary leukocytes. Therefore, in this regard, we have not shown direct evidence that integrin $\alpha 9\beta 1$ or CD11b is involved in increased neutrophil transmigration from circulating vessels into the alveolar space, the site of inflammation. However, our in vitro results show that recombinant IL-

Table 3. Correlation among IL-17A, integrin $\alpha 9\beta 1$, CD11b and CD18 in aspiration pneumonia patients and controls

	Integrin $\alpha 9\beta 1$	CD11b	CD18
IL-17A			
r value	0.47	0.11	0.07
p value	0.0033	0.3026	0.4915
Integrin $\alpha 9\beta 1$			
r value	–	–	–
p value	–	–	–
CD11b			
r value	0.14	–	–
p value	0.1790	–	–
CD18			
r value	0.28	0.21	–
p value	0.0065	0.0467	–

Pearson correlation coefficient was used to analyze the relationship between groups (pneumonia group on admission, pneumonia group on recovery and control group).

17A increased $\alpha 9\beta 1$ and CD11b expression levels in healthy human neutrophils and stimulated transmigration. Moreover, according to previous reports which showed increased pulmonary IL-17A in bacterial pneumonia, integrin $\alpha 9\beta 1$ activation stimulates inhibition of neutrophil apoptosis [24, 25, 28] and this might help progression to neutrophilic inflammation. Taking the results of our in vitro experiments and these previous reports together, serum IL-17A and integrin $\alpha 9\beta 1$ may collaborate to increase neutrophil transmigration.

Recently, increased pulmonary concentration of IL-17 was reported in an acid-aspiration pneumonia animal model [29]. In bacterial pneumonia, Th17 cells produce IL-17A after stimulation by IL-23. Pathogen-activated alveolar macrophages produce IL-23 and IL-23 stimulates Th17 cells [30–32]. After that IL-17 affects neutrophils, which are activated and migrate into alveolar space from vessels, and IL-22 is also produced by Th17 cells, playing a role in regulating lung inflammation [33]. Aujla et al. [34] reported that IL-22 played an important role in pulmonary mucosal host defense using an experimental bacterial pneumonia animal model. After receiving the stimulation of pathogens, IL-22 is upregulated and stimulates epithelial cells to produce chemokines for accumulating neutrophils into the site of pneumonia [34, 35]. In this study we could not detect significant elevation of IL-22 or IL-23 in any group, but this does not mean that they had not become elevated at the site of pulmonary inflammation. From previous reports, we speculate that it could be

possible that IL-22 and IL-23 were produced in local inflammatory circumstances and that IL-23 stimulated IL-17A production after pathogen activation of alveolar macrophages. The direct way to show this would be to measure IL-22 and IL-23 in human aspiration pneumonia lung tissue. In conclusion, the present study shows that under stimulation by IL-17A, expression of the $\beta 1$ integrin subfamily molecule, integrin $\alpha 9 \beta 1$, on neutrophils might be involved in the pulmonary inflammation of elderly aspiration pneumonia.

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Financial Disclosure and Conflicts of Interest

The authors declare that they have no competing interests.

References

- Marik PE, Kaplan D: Aspiration pneumonia and dysphasia in the elderly. *Chest* 2003;124:328–336.
- Kikuchi R, Watabe N, Konno T, Mishina N, Sekizawa K, Sasaki H: High incidence of silent aspiration in elderly patients with community-acquired pneumonia. *Am J Respir Crit Care Med* 1994;150:251–253.
- Taooka Y, Maeda A, Hiyama K, Ishioka S, Yamakido M: Effects of neutrophil elastase inhibitor on bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* 1997;156:260–265.
- Tamagawa K, Taooka Y, Maeda A, Hiyama K, Ishioka S, Yamakido M: Inhibitory effects of a lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Crit Care Med* 2000;161:1279–1284.
- Cowburn AS, Condliffe AM, Farahi N, Summers C, Chilvers ER: Advances in neutrophil biology: clinical implications. *Chest* 2008;134:606–612.
- Doerschuk CM, Mizgerd JP, Kubo H, Qin L, Kumasaka T: Adhesion molecules and cellular biomechanical changes in acute lung injury: Giles F. Filley lecture. *Chest* 1999;116:37S–43S.
- Doerschuk CM: Leukocyte trafficking in alveoli and airway passages. *Respir Res* 2000;1:136–140.
- Motosugi H, Quinlan WM, Bree M, Doerschuk CM: Role of CD11b in focal acid-induced pneumonia and contralateral lung injury in rats. *Am J Respir Crit Care Med* 1998;157:192–198.
- Bowden RA, Ding ZM, Donnachie EM, Petersen TK, Michael LH, Ballantyne CM, Burns AR: Role of $\alpha 4$ integrin and VCAM-1 in CD18-independent neutrophil migration across mouse cardiac endothelium. *Circ Res* 2002;90:562–569.
- Shang T, Yednock T, Issekutz AC: $\alpha 9 \beta 1$ Integrin is expressed on human neutrophils and contributes to neutrophil migration through human lung and synovial fibroblast barriers. *J Leukoc Biol* 1999;66:809–816.
- Tasaka S, Richer SE, Mizgerd JP, Doerschuk CM: Very late antigen-4 in CD18-independent neutrophil emigration during acute bacterial pneumonia in mice. *Am J Respir Crit Care Med* 2002;166:53–60.
- Palmer EL, Rüegg C, Ferrando R, Pytela R, Sheppard D: Sequence and tissue distribution of the integrin $\alpha 9$ subunit, a novel partner of $\beta 1$ that is widely distributed in epithelia and muscle. *J Cell Biol* 1993;123:1289–1297.
- Taooka Y, Chen J, Yednock T, Sheppard D: The integrin $\alpha 9 \beta 1$ mediates adhesion to activated endothelial cells and transendothelial neutrophil migration through interaction with vascular cell adhesion molecule-1. *J Cell Biol* 1999;145:413–420.
- Glynn P, Coakley R, Kilgallen I, O'Neill S: Neutrophil CD11b and soluble ICAM-1 and E-selectin in community acquired pneumonia. *Eur Respir J* 1999;13:1380–1385.
- Crowe CR, Chen K, Pociask DA, Alcorn JF, Krivich C, Enelow RI, Ross TM, Witztum JL, Kolls JK: Critical Role of IL-17RA in immunopathology of influenza infection. *J Immunol* 2009;183:5301–5310.
- Di Stefano A, Caramori G, Gnemmi I, Contoli M, Vicari C, Capelli A, Magno F, D'Anna SE, Zanini A, Brun P, Casolari P, Chung KF, Barnes PJ, Papi A, Adcock I, Balbi B: T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol* 2009;157:316–324.
- Miyashita N, Kawai Y, Akaike H, Ouchi K, Hayashi T, Kurihara T, Okimoto N: Clinical features and the role of atypical pathogens in nursing and healthcare-associated pneumonia (NHCAP): differences between a teaching university hospital and a community hospital. *Intern Med* 2012;51:585–594.
- Lim WS, van der Eerden MM, Laing R, Boersma WG, Karalus N, Town GI, Lewis SA, Macfarlane JT: Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. *Thorax* 2003;58:377–382.
- Fine MJ, Auble TE, Yealy DM, Hanusa BH, Weissfeld LA, Singer DE, Coley CM, Marrie TJ, Kapoor WN: A prediction rule to identify low-risk patients with community-acquired pneumonia. *N Engl J Med* 1997;336:243–250.
- Smith Hammond CA, Goldstein LB: Cough and aspiration of food and liquids due to oral-pharyngeal dysphasia: ACCP evidence-based clinical practice guidelines. *Chest* 2006;129:154S–168S.
- Teramoto S, Matsuse T, Fukuchi Y, Ouchi Y: Simple two-step swallowing provocation test for elderly patients with aspiration pneumonia. *Lancet* 1999;353:1243.
- Marcinkiewicz C, Taooka Y, Yokosaki Y, Calvete JJ, Marcinkiewicz MM, Lobb RR, Niewiarowski S, Sheppard D: Inhibitory effects of MLDG-containing heterodimeric disintegrin reveal distinct structural requirements for interaction of integrin $\alpha 9 \beta 1$ with VCAM-1, tenascin-C and osteopontin. *J Biol Chem* 2000;275:31930–31937.
- Werr J, Eriksson EE, Hedqvist P, Lindbom L: Engagement of $\beta 2$ integrins induces surface expression of $\beta 1$ integrin receptors in human neutrophils. *J Leukoc Biol* 2000;68:553–560.
- Saldanha-Gama RF, Moraes JA, Mariano-Oliveira A, Coelho AL, Walsh EM, Marcinkiewicz C, Barja-Fidalgo C: $\alpha 9 \beta 1$ integrin engagement inhibits neutrophil spontaneous apoptosis: involvement of Bcl-2 family members. *Biochim Biophys Acta* 2010;1803:848–857.
- Ross EA, Douglas MR, Wong SH, Ross EJ, Curnow SJ, Nash GB, Rainger E, Scheel-Toellner D, Lord JM, Salmon M, Buckley CD: Interaction between integrin $\alpha 9 \beta 1$ and vascular cell adhesion molecule-1 (VCAM-1) inhibits neutrophil apoptosis. *Blood* 2006;107:1178–1183.
- Nemzek JA, Fry C, Abatan O: Low-dose carbon monoxide treatment attenuates early pulmonary neutrophil recruitment after acid aspiration. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L644–L653.

- 27 Lin RY, Astiz ME, Saxon JC, Rackow EC: Altered leukocyte immunophenotypes in septic shock: studies of HLA-DR, CD11b, CD14 and IL-2R expression. *Chest* 1993;104:847–853.
- 28 Gupta SK, Vlahakis NE: Integrin $\alpha 9\beta 1$: unique signaling pathways reveal diverse biological roles. *Cell Adh Migr* 2010;4:194–198.
- 29 Walker MG, Yao LJ, Patterson EK, Joseph MG, Cepinskas G, Veldhuizen RAW, Lewis JF, Yamashita CM: The effect of tidal volume on systemic inflammation in acid-induced lung injury. *Respiration* 2011;81:333–342.
- 30 Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK: Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 2001;194:519–527.
- 31 Weaver CT, Hatton RD, Mangan PR, Harrington LE: IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 2007;25:821–852.
- 32 Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL: Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 2003;278:1910–1914.
- 33 Kudva A, Scheller EV, Robinson KM, Crowe CR, Choi SM, Slight SR, Khader SA, Dubin PJ, Enelow RI, Kolls JK, Alcorn JF: Influenza A inhibits Th17-mediated host defense against bacterial pneumonia in mice. *J Immunol* 2011;186:1666–1674.
- 34 Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, McAllister F, Edeal J, Gaus K, Husain S, Kreindler JL, Dubin PJ, Pilewski JM, Myerburg MM, Mason CA, Iwakura Y, Kolls JK: IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med* 2008;14:275–281.
- 35 Liu JZ, Pezeshki M, Raffatellu M: Th17 cytokines and host-pathogen interactions at the mucosa: dichotomies of help and harm. *Cytokine* 2009;48:156–160.