University of Louisville
Journal of Respiratory Infections

ORIGINAL RESEARCH

Neutrophil Function in Elderly Patients Hospitalized with Community-Acquired Pneumonia

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Abstract

Background: Advanced age is associated with immunosenescence as well as increased risk for poor outcomes during episodes of community-acquired pneumonia (CAP). Data on neutrophil function in hospitalized elderly patients with CAP is lacking. In this study we compared neutrophil function in elderly and non-elderly hospitalized patients with CAP.
Methods: Prospective study of healthy controls (HC) and patients hospitalized with CAP non-elderly (NE-CAP) and elderly (E-CAP). Blood samples were obtained on the day of hospitalization. The following neutrophil functional assays were performed: degranulation of secretory vesicles (CD35), degranulation of specific granules (CD66b), phagocytosis, and hydrogen peroxide (H_2O_2) production. The Mann-Whitney U-test was used to compare differences in neutrophil function.
Results: A total of 12 HC, 28 NE-CAP, and 12 E-CAP were evaluated. There were no significant differences between NE-CAP and E-CAP patients in regard to CD35 expression (p=0.465), CD66b expression (p=0.601), phagocytosis (p=0.654), or H_2O_2 production (p=0.541)
Conclusions: We failed to demonstrate any significant difference in neutrophil function in non-elderly versus elderly patients hospitalized with CAP in relation to membrane expression of CD35 and CD66b, phagocytosis, and respiratory burst. Abnormal neutrophil function is unlikely to be an important component of the immunosenescence described in elderly patients with CAP.

DOI: 10.18297/jri/vol1/iss1/3
Received Date: September 26, 2016
Accepted Date: November 31, 2016
Website: https://www.louisville.edu/jri

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1 Background

Elderly patients with community-acquired pneumonia (CAP) are at increased risk for poor clinical outcomes when compared to non-elderly patients. Advanced age is an independent risk factor for mortality present in several of the CAP severity scores[1]. Human aging is associated with a gradual decrease of immune function, defined as immunosenescence[2]. In the elderly, immunosenescence affects innate and adaptive immune responses. This may explain the increased susceptibility of the elderly to develop CAP, as well as the increased morbidity and mortality in these patients[3].

Neutrophils are the primary effector cells of the innate response and are the first line of defense against bacteria multiplying in the lung. During an episode of CAP, neutrophils recognize and bind immunoglobulin G molecules and complement proteins that coat the surface of bacteria in the alveoli. After binding to bacteria, killing is achieved by phagocytosis, the fusion of phagosomes with intracellular secretory granules, followed by degranulation and respiratory burst[4–6]. Even though there is a significant body of literature defining neutrophil function for several human conditions, none of these critical neutrophil functions have been characterized in patients with CAP.

Considering the importance of neutrophil function in the innate immune response during an episode of CAP and the poor clinical outcomes of CAP in the elderly, we hypothesized that decreased neutrophil function may be an important contributor to the immunosenescence described in the elderly. To test this hypothesis we compared neutrophil function in elderly and non-elderly hospitalized patients with CAP.

2 Methods

2.1 Study design and participants

This was a prospective observational study of hospitalized patients with CAP as well as healthy controls. Patients hospitalized with CAP were enrolled at the University of Louisville Hospital

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Table 1 Baseline characteristics of hospitalized patients with community-acquired pneumonia

Variable	Elderly ($\geq$ 65) n = 12 n = 28	Non-Elderly (<65) n (%) n (%)	p-value
Demographics			
Age, median (IQR)	78.5 (9)	53 (9.5)	
Male Gender	11 (92)	23 (82)	0.648
Past Medical History			
Smoking	0 (0)	16 (57)	0.001
Neurologic Diseases	1 (8)	2 (7)	1
Cerebrovascular Diseases	4 (33)	2 (7)	0.06
CHF	7 (58)	6 (22)	0.062
COPD	7 (58)	7 (26)	0.075
Diabetes mellitus	7 (58)	5 (19)	0.023
HIV	0 (0)	3 (11)	0.541
Acute Renal Diseases	2 (17)	1 (4)	0.229
Chronic Renal Diseases	3 (25)	2 (7)	0.159
Hepatic Diseases	0 (0)	2 (7)	1
Neoplastic Diseases	3 (25)	1 (4)	0.078
Hyperlipidemia	9 (75)	6 (21)	0.003
Statin Therapy	5 (42)	6 (21)	0.254
Immunosuppression	3 (25)	4 (14)	0.41
Vaccination History			
Previous Influenza vaccine	6 (50)	4 (14)	0.041
Previous Pneumococcal vaccine	8 (67)	5 (18)	0.008
Clinical, Laboratory and Radiological Findings			
Alter Mental Status	2 (17)	1 (4)	0.209
Pleural Effusion	4 (33)	8 (29)	1
ICU Admission	5 (42)	8 (29)	0.476
Fever (Temp $\geq$ 100°F)	5 (41.7)	16 (57.1)	0.580
White Blood Cell Count, Median (IQR)	14.0 (5.4)	11.4 (5.8)	0.092
Healthcare-Associated Pneumonia (HCAP)	3 (25)	7 (25)	1
Pneumonia Severity Index (PSI) $\geq$ 4	10 (83)	12 (43)	0.035
S. pneumoniae	4 (33)	5 (18)	0.411
Macrolide Therapy	4 (33)	10 (36)	1
Early Time to Clinical Stability (< 3 days)	7 (58)	19 (68)	0.72

and the Robley Rex Veterans Affairs Medical Center of Louisville from March 2011 to January 2012. The University of Louisville Human Subjects Program Protection Office and the Robley Rex Veterans Affairs Medical Center Institutional Review Boards approved this study prior to any data collection (Approvals #: 07.0182 and 0009, respectively).

2.2 Definition of CAP in the elderly

A patient was considered to have CAP if there was evidence of a new pulmonary infiltrate at chest radiograph at time of hospitalization plus at least one of the following criteria: 1) new or increased cough; 2) fever (>100°F) or hypothermia (<96°F); 3) changes in white blood cell count (leukocytosis, left shift, or leukopenia). A patient was defined as elderly if his/her age was $\geq$65 years.

2.3 Definition of healthy controls

Healthy controls were defined as blood donors meeting the following criteria: 1) negative history for chronic medical conditions, 2) not currently pregnant, 3) asymptomatic during the week previous to blood collection, and 4) not currently taking prescription or over-the-counter medication. Laboratory methods: Blood samples were obtained from CAP patients on the day of hospital admission. Venous blood was collected using sodium citrate Vacutainer®tubes. Following centrifugation at 300 $\times$ g for 10 min, the plasma was separated by aspiration, aliquoted and stored frozen at -80°C until assayed. The same process was followed for healthy control blood samples.

2.4 Neutrophil functional assays

Degranulation of secretory vesicles (CD35) and specific granules (CD66b) was determined by measuring plasma membrane expression, using flow cytometry. After incubation of whole blood with and without formyl-methionyl-leucyl phenylalanine for 5 min at 37°C, an ice-cold solution containing antibody FITC-conjugated monoclonal anti-CD35 or FITC-conjugated monoclonal anti-CD66b were added and the samples immediately placed at room temperature in the dark for 30 min. FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG1 were used as an isotype controls respectively. At the end of the incubation time the samples were mixed with lysis buffer, and incubated for 5 min at room temperature (RT), after which they were washed two times with 0.1% sodium azide and then resuspended in 1% paraformaldehyde, before being analyzed for fluorescence intensity by flow cytometry (mean channel fluorescence) on a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ). To measure phagocytosis and hydrogen peroxide production in phagosomes, whole blood samples were incubated with dichlorofluorescin diacetate for 10 min at 37°C. Fifty microliters of whole blood was sampled before, and 10 min later, the addition of 50 ml of opsonized, propidium iodide-labeled *Staphylococcus aureus* (final concentration 108 bacteria/ml). At the end of the incubation time, samples were mixed with lysis buffer, and allow to sit for 5 min at RT. The samples were then washed twice with 0.1% sodium azide, resuspended in 1% paraformaldehyde, and analyzed for fluorescence intensity by flow cytometry, excluding mononuclear cells.

2.5 Study groups and statistical analysis

Neutrophil function was evaluated for healthy controls (study group 1), hospitalized non-elderly patients with CAP (study group 2), and hospitalized elderly patients with CAP (study group 3). The Mann-Whitney U-test was used to compare differences in

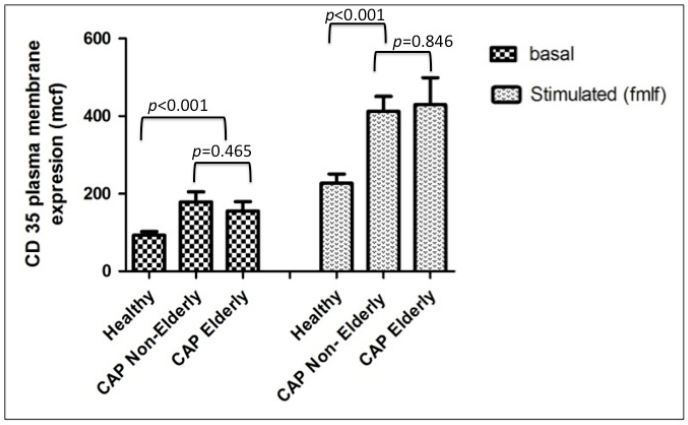

Fig. 1 Neutrophils plasma membrane expression of CD35 under baseline conditions and after stimulation with formyl-methionyl-leucyl phenylalanine (fmlf) for each of the study groups.

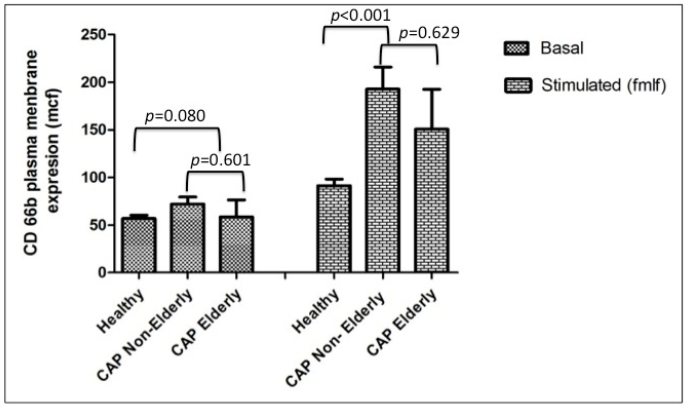

Fig. 2 Neutrophils plasma membrane expression of CD66b under baseline conditions and after stimulation with formyl-methionyl-leucyl phenylalanine (fmlf) for each study group.

neutrophil function between healthy controls (group 1) and hospitalized patients with CAP (groups 2 and 3 combined) as well as between non-elderly (group 2) and elderly (group 3) hospitalized patients with CAP.

3 Results

Neutrophil function was evaluated in 52 study participants, 12 healthy controls, 28 non-elderly hospitalized patients with CAP, and 12 elderly patients hospitalized with CAP. The baseline characteristics of non-elderly and elderly hospitalized patients with CAP are depicted in **Table 1**.

The neutrophil plasma membrane expression of CD35 under baseline conditions and after stimulation with formyl-methionyl-leucyl phenylalanine is depicted in **Figure 1**. A statistically significant increase in CD35 expression was seen for all CAP patients when compared to healthy donors under unstimulated and stimulated conditions ($p<0.001$ each). There were no significant differences in CD35 expression for non-elderly versus elderly patients with CAP under basal conditions ($p=0.465$) or after stimulation ($p=0.846$).

The plasma membrane expression of CD66b in neutrophils under baseline conditions and after stimulation with formyl-methionyl-leucyl phenylalanine is depicted in **Figure 2**. There were no differences between CD66b expression for all CAP patients versus healthy donors in unstimulated conditions ($p=0.080$), however a statistically significant increase in CD66b expression was seen for all CAP patients when compared to healthy donors under stimulated conditions ($p<0.001$). There were no significant differences in CD66b expression for non-elderly versus elderly patients with CAP under basal conditions ($p=0.601$) or after stimulation ($p=0.629$).

The neutrophil phagocytosis of opsonized propidium iodide-labeled *Staphylococcus aureus* for healthy donors, non-elderly hospitalized patients with CAP, and elderly hospitalized patients with CAP are depicted in **Figure 3**. No statistically significant differences were demonstrated among the study groups (CAP vs healthy $p=0.288$; CAP non-elderly vs elderly $p=0.654$).

The neutrophil hydrogen peroxide production in phagosomes for healthy donors, non-elderly hospitalized patients with CAP, and elderly hospitalized patients with CAP are depicted in **Figure 4**. A statistically significant increase in hydrogen peroxide production was demonstrated for CAP patients when compared to healthy

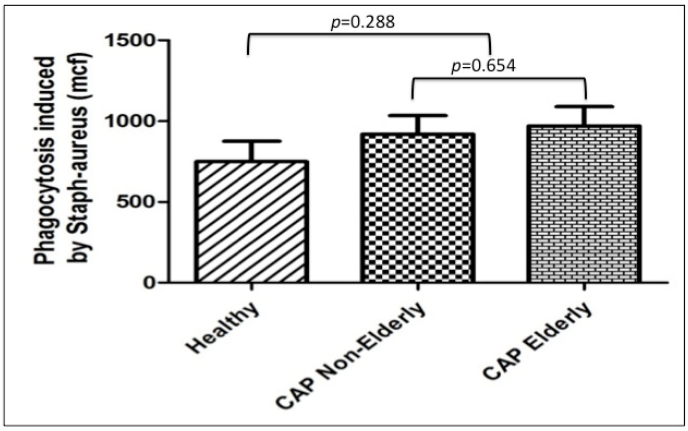

Fig. 3 Neutrophils phagocytosis of opsonized propidium iodide-labeled *Staphylococcus aureus* for each study group.

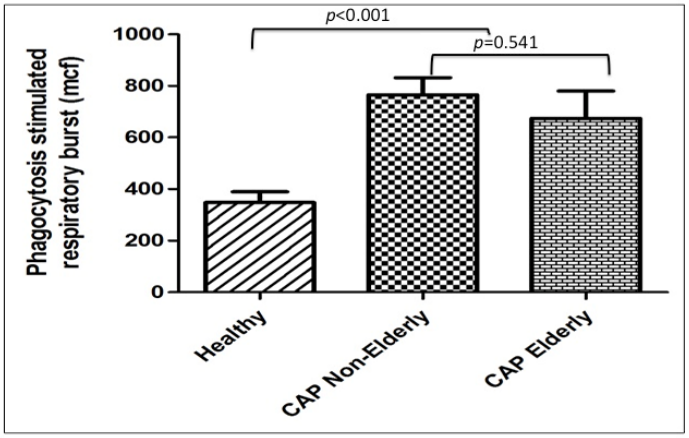

Fig. 4 Neutrophils hydrogen peroxide production in phagosomes for each of the study groups.

donors ($p<0.001$). There were no statistically significant differences in hydrogen peroxide production in non-elderly versus elderly hospitalized patients with CAP ($p=0.541$).

4 Discussion

In this study we failed to demonstrate any significant difference in neutrophil function in non-elderly versus elderly patients hospitalized with CAP in relation to membrane expression of CD35 and CD66b, phagocytosis, and respiratory burst.

Prior studies have documented that neutrophil precursors in the bone marrow, as well as the number of neutrophils present in the systemic circulation are not different in non-elderly and elderly adults[7,8]. Neutrophil chemotaxis is important to bring neutrophils from the pulmonary circulation into the alveoli. The process of neutrophil chemotaxis has been shown to be unaffected by age[9]. Once neutrophils migrate to the alveoli, the initial recognition and binding of opsonized bacteria is mediated by neutrophil surface receptors, including the complement receptor CR1 or CD35[10]. Degranulation of secretory vesicles and degranulation of specific granules are important neutrophil functions that are associated with membrane expression of CD35 and CD66b[10]. In our study, the expression of these membrane markers was similar in circulating neutrophils of non-elderly and elderly hospitalized patients with CAP. However, these two groups of CAP patients have a significant increase in the membrane markers CD35 and CD66b when compared to healthy controls after stimulation with formyl-methionyl-leucyl phenylalanine. This suggests that after maturation and release from the bone marrow, neutrophils of hospitalized patients with CAP are already primed in the peripheral circulation, and are ready to express membrane receptors once they arrive to the lung.

In our study we use opsonized propidium iodide-labeled *Staphylococcus aureus* as a marker of phagocytosis. These results indicate that the phagocytic ability of circulating neutrophils against *S. aureus* is not affected by age. Our data suggests that the phagocytic ability of circulating neutrophils in hospitalized patients with CAP is similar to that of healthy controls. It has been suggested that neutrophil phagocytosis may be pathogen specific. The fact that we found normal phagocytosis of *S. aureus* and a prior study indicated a reduced neutrophil phagocytosis of *E. coli* in elderly donors supports this concept[4].

After phagocytosis, stimulation of the respiratory burst with rapid release of reactive oxygen species is a crucial step for bacterial killing. In our study, we used production of hydrogen peroxide in

phagosomes as a marker of respiratory burst. We demonstrated that the level of phagocytosis stimulated respiratory burst is not decreased in non-elderly patients when compared to elderly patients with CAP. Our data is concordant with prior reports indicating that superoxide generation is not affected in neutrophils obtained from elderly donors[4].

The current study has several limitations. First, we only evaluated a limited number of hospitalized patients with CAP, which limits generalizability of our results. Second, we compared neutrophil function only in circulating neutrophils, but the activity of circulating neutrophils may not be a complete reflection of the activity of neutrophils once they migrate to the alveoli. Third, we evaluated the neutrophil membrane expression of two primary molecules, CD35 and CD66b, but neutrophils in elderly patients with CAP may fail to express other membrane receptors not evaluated in our study. Fourth, other neutrophil functions that may be associated with poor clinical outcomes, such as type of cell death, were not evaluated. Neutrophil death may occur in the form of apoptosis, netosis, pyroptosis, or necrosis[11]. Apoptosis and netosis are non-inflammatory programmed cell death types associated to beneficial effects. On the other hand, pyroptosis and necrosis are associated with increased inflammation that, in turn, may favor tissue damage and worse clinical outcomes[12].

Some aspects of neutrophil function in patients with CAP can be evaluated in neutrophils obtained from bronchoalveolar lavage samples as well as sputum samples. Our group recently reported an evaluation of neutrophil apoptosis from neutrophils obtained from sputum samples in a hospitalized patient with influenza CAP[13]. Future studies using these respiratory samples may give us a better understanding of neutrophil function at the alveolar level in hospitalized patients with CAP.

Beyond the pilot design, our study limitations include the assessment of neutrophil functions from the patient hospital admission day only and this assessment was in-vitro only.

In conclusion, this study demonstrates that some of the most critical neutrophil functions in patients with CAP are not decreased in the elderly, suggesting that abnormal neutrophil function is unlikely to be an important component of the immunosenescence described in elderly patients with CAP.

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