

# Variation of bronchoalveolar lymphocyte phenotypes with age in the physiologically normal human lung

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## Abstract

**Background**—Changes in T lymphocyte subsets have been observed in various forms of pulmonary disease. However, bronchoalveolar lymphocyte subsets have not been well characterised for healthy individuals differing in age. A study was undertaken to investigate the bronchoalveolar lavage (BAL) and peripheral blood lymphocyte subsets in clinically normal volunteers of two different age groups (19–36 and 64–83 years).

**Methods**—Bronchoalveolar lavage was performed on all individuals in both age groups and peripheral venous blood was drawn just prior to BAL. Bronchoalveolar cell profiles were characterised by morphological criteria, and cell surface antigen expression of lymphocytes was determined by flow cytometry.

**Results**—A significant increase in total BAL lymphocytes was observed for the oldest group compared with the youngest age group. Mean lymphocyte subset (CD4+/CD8+) ratios were significantly increased in BAL fluid from the older group compared with the younger group (mean (SE) 7.6 (1.5) vs 1.9 (0.2);  $p < 0.0001$ ). The increase in the BAL CD4+/CD8+ T cell ratio was mostly due to an increase in relative numbers of CD4+ lymphocytes, and the BAL CD4/CD8 ratio was disproportionately increased compared with peripheral blood in the older group. Increased expression of HLA-DR and CD69 on CD4+ T lymphocytes was observed in the oldest age group. Relative numbers of natural killer (NK) cells did not vary with age, and  $\gamma\delta$  T cells and CD5+ B cells were present in very low numbers in both age groups.

**Conclusions**—CD4+ T cells accumulate in air spaces of the lower respiratory tract with age in healthy adults and express increased amounts of HLA-DR and CD69 on their surfaces, suggesting a relative degree of CD4+ T lymphocyte activation for healthy older individuals who have normal lung function.

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Keywords: bronchoalveolar lavage; age; lung; lymphocyte; T cell

The decline of immune function with age has been well described for both experimental animals and humans.<sup>1</sup> A gradual decline in meas-

urable mammalian immune function begins early in adult life and corresponds with a gradual involution of the thymus gland. Age related changes include a shift towards increased CD4+ T cells of memory (CD44<sup>hi</sup>CD45RO+) phenotype and a decline in cells of the naive (CD44<sup>lo</sup>CD45RA+) phenotype.<sup>2-4</sup> Immunosenescence is also characterised by decreased proliferation of T cells in response to T cell receptor (TCR) stimulation,<sup>5,6</sup> and altered cytokine production in response to T cell activation.<sup>7,8</sup> Although humoral immune responses including antibody responses to antigens such as pneumococcal capsular polysaccharide also decline with advancing age,<sup>9</sup> monoclonal immunoglobulins and autoantibodies are seen with increased frequency in older humans or aged animals.<sup>10-13</sup> These findings suggest that the humoral immune system also loses functional capacity and becomes dysregulated with increasing age.

Although certain aspects of specific immunity in the elderly show a definite decline and a growing body of literature cites host pulmonary defence impairments in the elderly, little actual investigation has been conducted to characterise lymphocytes and immune cell function in the lower respiratory tract of older, clinically healthy individuals. Bronchoalveolar lavage (BAL) has been found to be a useful tool for the study of lower respiratory tract inflammation in many inflammatory pulmonary disorders and has provided much information on the pulmonary immune system and the complex interactions of immune cells and cytokines in the lung. However, BAL has been used infrequently to characterise lower respiratory tract secretions of older normal subjects.<sup>14,15</sup>

Because a pilot study suggested immune "dysregulation" in the lungs of older normal subjects with evidence of low level inflammation with increased lymphocytes and immunoglobulins in BAL fluid,<sup>15</sup> we expanded this study and used flow cytometry to characterise lymphocytes retrieved from the air spaces of the lung by BAL. We hypothesised that lymphocyte activation, autoimmune antibody production, complement activation, and/or immune complex formation may occur in the aging human lung in association with low grade inflammation. Therefore, in addition to characterising  $\alpha\beta$  TCR-bearing T lymphocytes and natural killer (NK) cells in BAL fluid, we measured  $\gamma\delta$  TCR-bearing T lymphocytes and CD5+ B lymphocytes which have been associated with autoimmune processes.<sup>16-18</sup> Because

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Table 1 Mean (SE) demographic and pulmonary function characteristics of subject groups

Parameter	Group I, age 19–36 (n = 19)	Group II, age 64–83 (n = 15)
Age	27 (1)	71 (1)
Sex (F:M)	7:12	8:7
FEV <sub>1</sub>	109 (2)	110 (3)
FVC	106 (2)	99 (3)
FEV <sub>1</sub> /FVC	82 (1)	70 (1)
FEF <sub>25–75</sub>	106 (4)	97 (7)

FEV<sub>1</sub> = forced expiratory volume in one second as percentage of normal predicted value; FVC = forced vital capacity as percentage of normal predicted value; FEV<sub>1</sub>/FVC = ratio of FEV<sub>1</sub> to FVC; FEF<sub>25–75</sub> = mean forced expiratory flow from 25% to 75% of FVC as percentage of predicted value.

activated lymphocytes express increased amounts of human leucocyte antigen (HLA) DR and CD69 and have been associated with autoimmune disorders,<sup>19–21</sup> expression of these surface antigens by CD4+ T cells was also examined.

## Methods

### SUBJECTS

Bronchoscopy with BAL was performed on two groups of normal volunteers stratified according to age (group I, 19–36 years, n = 19; group II, 64–83 years, n = 15). None of the subjects had ever smoked tobacco or other substances and all were physically active and in excellent health. None had a history of allergic rhinitis, other allergic disorders, or asthma, or a history of exposure to organic or inorganic dusts in the workplace or elsewhere, and no subjects had symptoms or signs of respiratory tract illness for at least four weeks prior to bronchoscopy. All subjects had normal physical examinations and normal spirometric pulmonary function test results. Study protocols were approved by the University of Wisconsin Center for Health Sciences Human Subjects Committee and informed written consent was obtained from all subjects.

### STUDY DESIGN

Candidates were screened and recruited from the Madison, Wisconsin area. Bronchoscopy was performed in the morning after overnight fasting in all subjects as previously described.<sup>15</sup> Peripheral venous blood was sampled just prior to bronchoscopy to obtain serum, plasma, and peripheral blood cells. No sedation was required for any subject. An Olympus fibreoptic bronchoscope was wedged in a subsegment of the right middle lobe and four 60 ml aliquots of sterile, non-pyrogenic, isotonic sodium chloride solution were instilled through the bronchoscope and recovered immediately by gentle hand suction.

The recovered aliquots of lavage fluid were pooled and the cellular and fluid phases of the pooled lavage fluid were separated by centrifugation. Total cell counts were determined with a haemocytometer and differential cell counts were obtained as previously described.<sup>15</sup> Heparinised peripheral blood was layered over a Polymorphprep (1.113, Nycomed, Oslo, Norway) cushion and centrifuged at 600g for 30 minutes at 22°C. Mononuclear cells at the

first interface were recovered and washed once in Hank's balanced salt solution and resuspended in media for subsequent labelling and flow cytometry.

To determine lymphocyte subsets in the BAL cellular fraction, cells were labelled with monoclonal fluorochrome conjugated antibodies directed against CD3 (Becton-Dickinson, San Jose, California, USA), CD4 (Olympus, Lake Success, New York, USA), CD5 (Becton-Dickinson), CD8 (Olympus), CD11c (Becton-Dickinson), CD16 (Becton-Dickinson), CD19 (Caltag, South San Francisco, California, USA), CD45 (Becton-Dickinson), CD56 (Olympus), anti-DR (Becton-Dickinson), anti- $\gamma\delta$  T cell receptor ( $\gamma\delta$ -TCR; Becton-Dickinson), CD69 (Becton-Dickinson), and IgM (Caltag, Burlingame, California, USA) as previously described.<sup>15</sup> Appropriate fluoroconjugates (fluorescein and phycoerythrin) were used to allow simultaneous dual labelling of bronchoalveolar or peripheral blood mononuclear cells when desired.  $1 \times 10^5$  cells in 100  $\mu$ l FACS buffer (2% bovine serum albumin, 0.2% NaN<sub>3</sub> in phosphate buffered saline) were incubated for 30 minutes with the appropriate FITC and/or PE conjugated antibodies. Red blood cells were lysed using Becton-Dickinson lysing solution for 10 minutes. The cells were then centrifuged, washed with FACS buffer, and fixed with FACS FIX (1% paraformaldehyde, 1.08% sodium cacodylate, 0.66% sodium chloride) for 30 minutes at 4°C. Flow cytometry was performed within 36 hours of fixation to detect and quantitate labelled cells.

### ANALYSIS OF DATA

Independent *t* tests, analysis of variance, and multivariate analysis and multiple regression analysis were performed using a database statistics package for microcomputers (Abstat 4.1; Anderson-Bell, Parker, Colorado, USA). Values are expressed as mean (SE) unless otherwise stated.

## Results

Characteristics of the study subjects are given in table 1. The mean percentage of lymphocytes in BAL fluid on differential counting (table 2) was significantly increased in the older age group compared with the younger group (range 3.4–42.7%, median 12.0% vs 1.7–18.3%, median 8.0%), as were the mean total lymphocytes/ml BAL fluid (range  $2.7$ – $63.6 \times 10^3$ /ml, median  $18.3 \times 10^3$ /ml vs  $2.1$ – $25.1 \times 10^3$ /ml, median  $9.8 \times 10^3$ /ml). Total cells/ml BAL fluid were also significantly increased in the older age group (table 2). However, the percentage of lymphocytes on cell differential counting and the concentration of lymphocytes in the BAL fluid did not correlate significantly with age for individuals within the two age groups.

The ratio of CD4+ to CD8+ T lymphocytes in BAL fluid was significantly increased in the older group (range 1.14–20.2, median 6.19) compared with the younger group (range 0.43–6.85, median 1.59; table 3) and confirmed the results of a previously reported cohort of subjects.<sup>15</sup> When the relative total

Table 2 Mean (SE) bronchoalveolar lavage cellular analysis

Parameters	Group I	Group II	p value†	A	CI
Volume retrieved (ml)	163 (2)	135 (8)	<0.001	28	13 to 43
Total cells ( $\times 10^3$ /ml BAL fluid)	122 (9)	163 (25)	0.11	41	-7 to 89
% Macrophages	90 (1)	80 (3)	<0.01	10	4.3 to 15.7
Macrophages ( $\times 10^3$ /ml BAL fluid)	110 (9)	129 (21)	NS	19	-23 to 61
% Lymphocytes	8.3 (0.9)	17.0 (3.3)	<0.05	8.7	2.7 to 14.7
Lymphocytes ( $\times 10^3$ /ml BAL fluid)	10.2 (1.5)	28.7 (6.2)	<0.01	18.5	6.6 to 30.4
% Neutrophils	1.2 (0.2)	2.7 (0.7)	0.03	1.5	0.2 to 2.8
Neutrophils ( $\times 10^3$ /ml BAL fluid)	1.5 (0.2)	4.5 (1.6)	<0.05	3.0	0.2 to 5.8
% Eosinophils	0.3 (0.1)	0.2 (0.1)	NS	0.1	-0.2 to 0.4
Eosinophils ( $\times 10^3$ /ml BAL fluid)	0.3 (0.1)	0.3 (0.1)	NS	0	-0.3 to 0.3
Total protein ( $\mu$ g/ml)	77 (4)	111 (8)	<0.001	34	18 to 50

\*BAL fluid = bronchoalveolar lavage supernatant fluid.

†p value for group I versus group II (two tailed Student's independent t test).

Table 3 Lymphocyte subsets in BAL fluid and peripheral blood. All analyses were performed on 19 subjects in group I and 15 subjects in group II with the exception of CD4+/CD69+ lymphocytes (n = 6 for group I and n = 7 for group II)

Parameter	Group I		Group II	
	BAL fluid	Blood	BAL fluid	Blood
%CD3+	79.7 (3.3)	70.1 (1.9)	79.8 (3.0)	65.7 (2.9)
%CD19+	2.2 (0.4)	11.4 (0.8)	1.2 (0.2)*	10.7 (1.0)
%CD16+/56+	3.4 (0.5)	11.8 (1.3)	3.1 (0.5)	12.8 (1.3)
%CD4+	46.4 (2.9)	40.3 (1.4)	68.4 (4.2)*	39.5 (2.8)
%CD8+	31.6 (3.5)	31.8 (1.4)	12.6 (1.7)*	26.0 (2.6)
CD4/CD8 ratio	1.9 (0.3)	1.4 (0.1)	7.6 (1.5)†	2.0 (0.4)*
% $\gamma\delta$ TCR+	2.9 (0.3)	4.5 (0.5)	0.97 (0.19)*	1.8 (0.3)
%CD5+/IgM+	0.93 (0.20)	0.19 (0.03)	1.26 (0.23)	0.17 (0.06)
%CD4+/DR+	20.3 (2.1)	2.9 (0.2)	33.8 (3.8)*	4.1 (0.4)
%CD4+/CD69+	33.8 (3.5)	8.3 (1.6)	51.5 (7.0)*	4.8 (1.2)

\*p<0.05 vs group I; †p<0.01 vs group I.

numbers of CD4+, CD8+, and CD4+/HLA-DR+ T cells in BAL fluid were calculated by multiplying the percentage of cells positive by the total lymphocytes/ml BAL fluid the numbers of CD8+ lymphocytes in BAL fluid were similar in the two age groups (group I,  $4.7 (0.7) \times 10^3$ /ml; group II,  $4.4 (1.0) \times 10^3$ /ml). However, absolute numbers of CD4+ lymphocytes (group I,  $6.4 (0.7)$ ; group II,  $18.5 (3.2) \times 10^3$ /ml) and CD4+/HLA-DR+ lymphocytes (group I,  $2.6 (0.5)$ ; group II,  $11.8 (3.3)$ ; p<0.01) were significantly increased (fig 1). The CD4/CD8 ratio in BAL fluid was considerably higher than the peripheral blood CD4/CD8 ratio for the older age group than for the young age group. No significant difference in CD4/CD8 T lymphocyte ratios for the entire group or for the youngest or oldest age group could be found for females vs males, nor was there a significant correlation of the CD4/CD8 ratio with age within each age group.

Relatively few CD19+ B cells were present in either group and these were lower in the older age group (table 3). Relative percentages of natural killer cells (CD16+/CD56+) were similar for the two groups. Few  $\gamma\delta$  T cells or CD5+ B cells (CD5+/IgM+) were identified in the BAL fluid in either age group, and the relative percentage of  $\gamma\delta$  T cells was lower for the older age group than for the younger. However, absolute numbers of  $\gamma\delta$  lymphocytes/ml BAL fluid were similar for the two age groups and the number of CD5+ B cells/ml BAL fluid was increased in the oldest age group. Dual labelling of lymphocytes with anti-CD4 and anti-CD69 revealed increased relative numbers of CD4+/CD69+ cells in the older subjects (table 3).

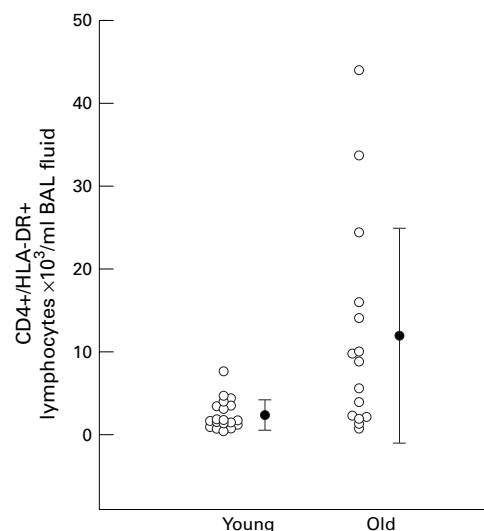


Figure 1 Mean (SD) total CD4+/HLA-DR+ T lymphocytes/ml BAL fluid in young and old subjects (p<0.01, independent two tailed t test).

## Discussion

Although lymphocytes and lymphocyte subsets retrieved from the lung by BAL have been extensively studied in various disease states and to some degree in normal individuals,<sup>22-24</sup> little information exists concerning lower respiratory tract air space lymphocytes and lymphocyte subsets as a function of age for healthy subjects with normal lung function. Previous studies have shown that the CD4/CD8 T lymphocyte ratio in human peripheral blood tends to increase with advancing age,<sup>25-26</sup> and studies in mice<sup>27</sup> and humans<sup>28</sup> suggest that the CD4/CD8 ratio in peripheral blood is genetically controlled. Our data suggest that CD4/CD8 T cell ratios in the lower respiratory tract air spaces of normal subjects increase with advancing age, and this increase is greater in magnitude for older individuals than that of the peripheral blood CD4/CD8 ratio. This age related increase in CD4 lymphocytes and CD4/CD8 ratio in healthy individuals stands in contrast to the increased numbers of CD8 T cells and depressed CD4/CD8 ratios observed in older smokers who develop chronic obstructive lung disease.<sup>28</sup> The relative increase in CD4+ lymphocytes which we found for older healthy subjects probably represents an accumulation of memory cells and primed T cells which generally bear a high density of the CD45RO antigen on their surfaces<sup>29</sup> due to cumulative antigenic stimulation at mucosal surfaces.

The function of  $\gamma\delta$  T cell and CD5+ B cell subsets is not well defined but both may play important roles in mucosal immunity against certain infectious agents,<sup>30-31</sup> in addition to their association with autoimmune phenomena. We found that relative numbers of  $\gamma\delta$  T cells and CD5+ B cells were very low in BAL fluid from both young and old subjects, although the mean concentration of CD5+ B cells/ml BAL fluid was somewhat increased for the older age group. Expression of HLA-DR and CD69 by CD4+ lymphocytes was increased in the older subjects which suggests a

relative degree of activation of this subset. This finding was of particular interest because HLA class II positive T cells accumulate in tissues affected by autoimmune disorders and may be involved in the immunopathogenesis of autoimmune disorders.<sup>21</sup>

The significance of age associated changes in lymphocyte populations for lung function or coordinated immune responses to infectious agents is unknown. Increased numbers of CD4+/HLA-DR+ T lymphocytes may reflect a low grade inflammatory or autoimmune reaction in the lung in many asymptomatic, clinically normal, older individuals. However, increased numbers of memory cells and increased antibody concentrations on epithelial surfaces may actually be beneficial in protecting an aging, more susceptible host against infectious agents. Two studies in aging elderly populations have shown that low CD4+ T cell counts in peripheral blood predict an increased risk of mortality,<sup>32</sup> and our results may be biased towards increased relative numbers of CD4+ T cells in BAL fluid and peripheral blood due to the selection of a healthy study population with normal lung function.

In summary, we have shown that absolute numbers of lymphocytes and CD4/CD8 T lymphocyte ratios increase in air spaces of the lower respiratory tract in association with advancing age in healthy subjects, and that increased numbers of CD4+ T cells in BAL fluid express the activation markers CD69 and HLA-DR. We speculate that activated lymphocyte subsets may play a role in age related low grade inflammation in the aging lung and contribute to alterations in lung matrix and function which are associated with the aging process. Our data show that care must be taken to consider age when analysing lower respiratory tract secretions retrieved by BAL in study protocols because lymphocyte numbers and subsets can vary with advancing age in normal subjects.

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