CD4+ and CD8+ T lymphocytes in lung tissue of NSIP: Correlation with T lymphocytes in BALF

Ling Qin b,f, Wen-Ze Wang d,f, Hong-Rui Liu d, Wen-Bing Xu a, Ming-Wei Qin e, Zhu-Hua Zhang e, Yi Xiao a, Wen-Jie Zhen c, Ju-Hong Shi a,*

a Department of Pulmonary Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
b Department of Internal Medicine, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
c Department of Rheumatology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
d Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
e Department of Radiology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China

Received 12 May 2012; accepted 24 September 2012
Available online 22 October 2012

KEYWORDS
Non-specific interstitial pneumonia; CD4+; CD8+; T lymphocytes; Surgical lung biopsy; Bronchoalveolar lavage

Summary
Background: Nonspecific interstitial pneumonia (NSIP) is characterized by the interstitial infiltration of T lymphocytes (TLs). Bronchoalveolar lavage fluid (BALF) has been used to analyze the inflammatory cells infiltrating in lung. The controversy about whether the BALF cellular profile reflects TLs in lung tissue still persists. Some studies found a positive correlation of cell composition between BALF and lung tissue, but others gave opposite conclusion.
Objective: To investigate CD4+ and CD8+ T lymphocytes distribution in lung tissue of NSIP and the relationship with TLs in bronchoalveolar lavage.
Methods: Thirty-seven patients diagnosed as NSIP were included. The pathological and BALF data were reviewed. The characteristics of TLs infiltration in different lung regions were investigated.

Abbreviations: NSIP, nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; BALF, bronchoalveolar lavage fluid; IPF, idiopathic pulmonary fibrosis; ILD, interstitial lung disease; TLs, T lymphocytes; ESR, erythrocyte sedimentation rate; TLC, total lung capacity; DLCO, diffusion lung capacity for carbon monoxide; SSC, systemic scleroderma; pSS, primary sjogren’s syndrome; PM, polymyositis; HRCT, high resolution computed tomography.

* Corresponding author. Tel./fax: +86 10 65295527.
E-mail addresses: juhong_shi@hotmail.com, juhong_shi@yahoo.com (J.-H. Shi).
† The first two authors, Ling Qin and Wen-Ze Wang, contribute equally to this work.

0954-6111/S - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rmed.2012.09.021
CD4+ and CD8+ T lymphocytes in lung tissue of NSIP

Results: The study included 28 women. The median age was 48 years. In lung tissue, CD4+ and CD8+ lymphocytes (counts/0.1mm2) were separately accounted in lymphoid follicle region (156.51 ± 90.70 vs 85.30 ± 43.75), small blood vessel region (66.58 ± 31.99 vs 58.43 ± 30.24), interstitial region (37.60 ± 19.40 vs 47.12 ± 33.42) and small airway region (26.59 ± 17.04 vs 40.18 ± 34.02). CD4+/CD8+ ratios in lymphoid follicle and small vessel > 1, in interstitium and small airway <1. The number of CD8+ lymphocytes in BALF was correlated with CD8+ lymphocytes around small airway (r = 0.360, p = 0.029) and in interstitial region (r = 0.451, p = 0.005). CD4+/CD8+ ratio in BALF was correlated with that in small airway region (r = 0.437, p = 0.007) and interstitial region (r = 0.468, p = 0.003). Conclusions: In NSIP, T lymphocytes were distributed in different regions of lung tissue. The CD8+ T lymphocytes and CD4+/CD8+ ratio in BALF reflect those in interstitium regions and around small airway of the lung.

© 2012 Elsevier Ltd. All rights reserved.

Introduction

Nonspecific interstitial pneumonia (NSIP) is an injury pattern that can be found in many clinical settings. It can be associated with many conditions such as connective tissue disease, drug exposure and hypersensitivity pneumonia.1 This histologic pattern is characterized by infiltration of inflammatory cells in the interstitium, consisting of lymphocytes and plasma cells. Infiltrating lymphocytes, predominantly CD4+ T lymphocytes, with an elevated CD4+/CD8+ T cell ratio were found in NSIP patients.2,3 The distribution of T lymphocyte subsets reveals that CD4+ TLs are observed mainly inside and around lymphoid follicles and in the thick fibrotic wall of reconstructed alveoli, while CD8+ TLs are diffusely distributed, especially in relatively thin alveoli.4

Bronchoalveolar lavage fluid (BALF) has been used to analyze the inflammatory cells infiltrated in alveolar wall and around small airways.5-7 When BAL is used in conjunction with comprehensive clinical information and HRCT, BAL nucleated immune cell patterns can frequently provide useful information for diagnostic evaluation and lessen the need to proceed to more invasive procedures, such as surgical lung biopsy.8

However, controversy always exists about whether the BALF cellular profile reflects cell composition in lung tissue. Some studies9-14 have shown a positive correlation of composition between BALF and the lung tissue and BALF findings may consider as a predictor of progression or treatment response. Whereas other studies have given an15-18 opposite conclusion, BAL fluid components, cells in particular, are not correlated well with activity of disease nor for monitoring disease progress or response to treatment.19

Is the distribution form of lymphocyte subsets in the lung tissue obtained by surgical lung biopsy different from that in BALF? Why are the studies about the correlation of T-lymphocyte subsets between lung tissue and BALF controversial?

In order to better understand the underlying reasons of these inconsistencies, this study was performed to evaluate the distribution features of T lymphocyte subsets in NSIP lung tissue and the relationship of lymphocyte subsets distribution between the lung tissue and BALF.

Material and methods

Subjects

Between April 2003 and August 2010, ninety-six patients from Peking Union Medical College Hospital (PUMCH) were diagnosed histopathologically as NSIP by surgical lung biopsy. Thirty-seven of these ninety-six NSIP patients, who had the results of lymphocytes analysis of BALF, were enrolled in this study. None of these Thirty-seven NSIP patients had the history of occupational inhalation of dust or virulent drug exposure. Hypersensitivity pneumonitis was excluded according to patient’s exposure history, serum antibodies tests to the offending antigen.20,21 None of them were treated with corticosteroid and immunosuppressive at the time of bronchoscope examination and surgical lung biopsy. BALF and TBLB were done at first and after the infectious disease was excluded, surgical lung biopsy was performed within 2 weeks. Their clinical features, radiological images, BALF lymphocyte subsets and pathological findings were reviewed and analyzed retrospectively.

Histology of lung tissue

Surgical lung biopsy specimens were reviewed by two senior pathologists independently and the consensus was reached for the histologic pattern. Thirty-seven cases were subclassified according to criteria proposed by Katzenstein and Fiorelli22 as cellular, mixed and fibrotic pattern (Supplement materials Table 1). Small airways were defined as those with the internal diameter less than 2 mm and without cartilage.23 Small blood vessels were defined as those with the internal diameter no more than 100 µm.22

Immunohistochemistry of lung tissue

Lung biopsy specimens were fixed in 10% neutral buffered formalin solution, cut into slices, embedded in paraffin, and sectioned at 4 µm paraffin thickness for histologic evaluation. Paraffin sections were immunohistochemically stained with anti-CD4 antibody (clone SP35, Zeta), and anti-CD8 antibody (clone EP1150, Zeta), according to the labeled Streptavidin—Biotin Complex method. The sections...
were deparaffinized and rehydrated with Tris-Buffered Saline (TBS: 0.005 M Tris, 0.15 M NaCl), pH = 7.6 for 10 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. Then they were washed in TBS and incubated with primary antibodies at the appropriate dilutions for 1 h CD4 and CD8 staining was performed on the same tissue sections.

The number of positively stained cells with dark brown staining were analyzed by the NanoZoomer 2.0-RS slide scanning system (Hamamatsu Photonics KK; Japan) and Anymicro DSS Pro image analysis system (YuTianShiJiWeiYe INC; Beijing, China). Four microscopic regions (follicles, small blood vessels, interstitium and small airways) were analyzed. Each biopsy slide contains 4 microscopic regions of above mentioned. CD4 and CD8 images were obtained in the same region of the slide (Fig. 1). At least 6 high-power fields (magnification × 20; analysis area, approximately 0.162 mm²) were randomly selected for each region and used for positive cell counting.

Analysis of lymphocyte subsets from BALF

Thirty-seven patients with NSIP underwent bronchoscope examination with electric video bronchoscope (Olympus Co, Tokyo, Japan) wedged into a segmental bronchus of the right middle lobe or lingula of the left upper lobe. One hundred milliliters of sterilized saline were injected gently by a syringe through the bronchoscope and collected by deep inspiration according to the guidelines for measurement of acellular components and standardization of BAL. The BALF was pooled, measured in volume, and then filtered through nylon sterile gauze to remove mucus. The BALF was centrifuged at 400 g 4 °C for 10 min to separate cells from fluids. The total cell count was evaluated on an aliquot of the pooled fluid using a Neubauer counting chamber. The cells were washed twice with phosphate-buffered saline solution and differential cell counts were performed with May-Grunwald-Giemsa staining. To evaluate the cell subsets, lymphocytes were stained with anti-CD3, anti-CD4 and anti-CD8 monoclonal antibodies coupled to fluorescein isothiocyanate, and cellular fluorescence was measured with a FACS calibur flow cytometer.

Treatment and follow up

At the initiation of treatment, all patients received a course of oral prednisolone, starting at 0.5 mg/kg/d for one month which was then tapered every 3 weeks to 5–7.5 mg/d. The total length of treatment was 12–18 months. Cyclophosphamide was administered at the beginning with steroid therapy at the dose of 100 mg/d given orally for 18 months in patients diagnosed as CTD-NSIP. Patients underwent routine examinations every 3 month after surgical lung biopsy, and then once a year in the following years in Interstitial Lung Disease Clinic of PUMCH.

Statistical analysis

All expressed values are mean ± SD. T test was used for analysis of normal distribution samples. Wilcoxon Test and Kruskal–Wallis Test were used to compare two or more

Figure 1 Distribution of T lymphocytes in different regions of lung tissue: CD4⁺ and CD8⁺ TLs decreased gradually in order of follicle, small blood vessel, interstitial and around small airways regions.
non-normal distribution samples, respectively. Correlation coefficients were calculated using the Spearman rank method. Probability value was obtained from 2-sided tests, with a statistical significance of \( p < 0.05 \). SPSS 15.0 (SPSS for Windows, version 15.0 [SPSS Inc., Chicago, Illinois, USA]) was used for statistical analysis.

This study was approved by the Peking Union Medical College Institutional Review Board.

Patients who did not authorize the use of their medical records for research were excluded from this study.

## Results

### Clinical features and laboratory findings

The mean age of 37 patients was 48.9 ± 10.5 years (range from 23 to 68 years) and 28/37 (75.7%) patients were women. There were 31/37 (83.8%) nonsmokers, 4/37 (10.8%) ex-smokers and 2/37 (5.4%) current smokers. Five NSIP patients were secondary to CTD, including 3 primary sjögren’s syndrome, 1 systemic scleroderma, and 1 polymyositis. The clinical features and laboratory results of 37 patients were summarized in Table 1. The duration of respiratory symptoms from onset to lung biopsy ranged from 0.8 to 72 months (median 6 months). All the patients were followed up for a median period of 40 months (range from 15 to 109 months). During follow-up, 4/37 (10.8%), including 2 patients with mixed NSIP and 2 with fibrotic NSIP died of progression of the disease.

### T lymphocyte subsets distribution in different regions of lung tissue

Distribution of CD4\(^+\) T lymphocytes was shown in Fig. 1. The counts/0.1 mm\(^2\) of CD4\(^+\) T lymphocytes (CD4\(^+\) TLs) decreased gradually in order of follicle, small blood vessel, interstitial and small airway regions (Supplement materials Table 2). The differences of cell counts between every two different regions were statistically significant (Fig. 2A, all \( p < 0.001 \)).

Distribution of CD8\(^+\) T lymphocytes was shown in Fig. 1. It had similar trend to the distribution of CD4\(^+\) T lymphocytes (Supplement materials Table 2). Lymphocytes in follicles were much more than those in each of the other three regions (Fig. 2B, all \( p < 0.001 \)).

### The distribution of CD4\(^+\) and CD8\(^+\) T lymphocytes in different pathologic patterns of NSIP

According to pathologic criteria of NSIP, 15 cellular NSIPs, 19 mixed NSIPs and 3 fibrotic NSIPs were diagnosed and analyzed. The patients were divided into two groups: cellular and mixed/fibrotic groups (only 3 patients in fibrotic group, so we combined mixed and fibrotic NSIPs in one group). Comparing with mixed/fibrotic NSIPs, we found that CD4\(^+\) TLs infiltrating around small blood vessel and in interstitial region were more prominent in cellular NSIPs. CD8\(^+\) TLs infiltrating in lung tissue of cellular NSIPs were also more than those in mixed/fibrosis NSIPs in each of the studied regions (Table 2). Since CD4\(^+\) TLs decreased more prominent than CD8\(^+\) TLs in order of follicle, small blood vessel, interstitial and small airway regions, CD4\(^+\)/CD8\(^+\) ratios in cellular pattern decreased more sharply than those in mixed/fibrotic pattern, with the ratio <1 in cellular pattern and >1 in mixed/fibrotic pattern in the regions of interstitial and small airway. However, the differences of CD4\(^+\)/CD8\(^+\) ratios between cellular group and mixed/fibrotic group were not statistically significant in any of the four regions (Table 2).

### Relationship between lung tissue and BALF for CD4\(^+\) and CD8\(^+\) T lymphocytes and CD4\(^+\)/CD8\(^+\) ratio

The relationship between T lymphocyte subsets in BALF and those infiltrating in lymphoid follicles, around small blood vessels, interstitial region or around small airways were analyzed (Supplement materials Table 2) respectively. CD8\(^+\) TLs in BALF were significantly correlated with those around small airways (Fig. 3A) and with those in interstitial region (Fig. 3B), but not with those around small blood vessels and in lymphoid follicles. CD4\(^+\) TLs in BALF were not correlated with those in any of the four study regions.

The relationship of CD4\(^+\)/CD8\(^+\) ratio between BALF and each of the four study regions were analyzed (Supplement materials Table 2). The relationship was significant in interstitial region (Fig. 4A) with \( p < 0.05 \) and around small blood vessels (Fig. 4B) or around small airways with \( p < 0.001 \).
CD4+ T lymphocytes were mainly infiltrated inside lymphoid follicle and perivascular region. In contrast, CD8+ T lymphocytes were diffusely distributed in lung tissue with the highest quantity in lymphoid follicle and similar distribution among interstitium, small vessel and small airway regions. The different distribution patterns of CD4+ and CD8+ TLs suggested that they might play different roles in the process of the lung injury.25,26 CD4+ T lymphocytes, as T helper cells, might express different chemokine receptors which could facilitate themselves move to the various tissues, especially to lymphoid follicles and small vessels. CD8+ T lymphocytes, as the effective cells, can directly combine with the class I antigens and remove pathogen or foreign body. It was more convenient and important for CD8+ T lymphocytes distributing diffusely in the lung tissue to keep the balance of immune defence function.27,28

Variable CD4+/CD8+ ratios in lung tissue of NSIP

Shimizu29 found that CD4+ TLs were the main inflammatory cells in ILD associated with CTD (CTD-ILD), while CD8+ TLs, in IPF, CD4+/CD8+ ratio of CTD-ILD was lower than that of IPF.29,30 According to CD4+/CD8+ ratio in the lung tissue, Fireman32 divided NSIP patients into two categories, patients with high CD4+/CD8+ ratio (>1) and with low CD4+/CD8+ ratio (<1). He considered that the

<table>
<thead>
<tr>
<th>Cellular pattern (n = 15)</th>
<th>Mixed/fibrotic pattern (n = 22)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD4+ T lymphocytes of tissue (counts/0.1 mm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoid follicle region</td>
<td>168.64 ± 93.14</td>
<td>148.24 ± 90.21</td>
</tr>
<tr>
<td>Small blood vessel region</td>
<td>86.82 ± 33.70</td>
<td>52.77 ± 22.48</td>
</tr>
<tr>
<td>Interstitial region</td>
<td>47.76 ± 20.75</td>
<td>30.67 ± 15.29</td>
</tr>
<tr>
<td>Small airway region</td>
<td>30.25 ± 17.97</td>
<td>24.09 ± 16.33</td>
</tr>
<tr>
<td><strong>CD4+ T lymphocytes of BALF (10⁶/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.79 ± 0.75</td>
<td>0.97 ± 0.96</td>
</tr>
<tr>
<td><strong>CD8+ T lymphocytes of tissue (counts/0.1 mm²)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoid follicle region</td>
<td>109.67 ± 58.09</td>
<td>68.68 ± 17.82</td>
</tr>
<tr>
<td>Small blood vessel region</td>
<td>66.93 ± 36.72</td>
<td>35.82 ± 16.05</td>
</tr>
<tr>
<td>Interstitial region</td>
<td>61.98 ± 44.81</td>
<td>36.98 ± 17.61</td>
</tr>
<tr>
<td>Small airway region</td>
<td>57.22 ± 44.87</td>
<td>28.56 ± 17.12</td>
</tr>
<tr>
<td><strong>CD8+ T lymphocytes of BALF (10⁶/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.63 ± 4.23</td>
<td>1.07 ± 1.23</td>
</tr>
<tr>
<td><strong>CD4+/CD8+ ratio of tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoid follicle region</td>
<td>1.69 ± 0.70</td>
<td>2.36 ± 1.67</td>
</tr>
<tr>
<td>Small blood vessel region</td>
<td>1.43 ± 0.48</td>
<td>1.70 ± 0.94</td>
</tr>
<tr>
<td>Interstitial region</td>
<td>0.89 ± 0.91</td>
<td>1.04 ± 0.95</td>
</tr>
<tr>
<td>Small airway region</td>
<td>0.67 ± 0.50</td>
<td>1.14 ± 1.04</td>
</tr>
<tr>
<td><strong>CD4+/CD8+ ratio of BALF</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.57 ± 0.15</td>
<td>1.12 ± 1.30</td>
</tr>
</tbody>
</table>
CD4⁺ and CD8⁺ T lymphocytes in lung tissue of NSIP

Figure 3 T lymphocyte profile relationship between tissue and BALF disclose positive relations were found between CD8⁺ TLs in small airway and those in BALF (A), interstitial CD8⁺ TLs and those in BALF (B), CD4⁺/CD8⁺ ratios in small airway and that in BALF (C), interstitial CD4⁺/CD8⁺ ratios and CD4⁺/CD8⁺ ratios of BALF (D), respectively.

high CD4⁺/CD8⁺ ratio was the feature of cellular pattern and at the early stage of the disease, while the low CD4⁺/CD8⁺ ratio was associated with fibrotic pattern and at the late stage of the lung injury. Our results showed that even in the same patient with cellular NSIP pattern, CD4⁺/CD8⁺ ratio was quite different in different regions. The CD4⁺/CD8⁺ ratios in lymphoid follicle and small blood vessel regions were higher (>1) than those in interstitial and small airway regions (<1). These results might explain the different descriptions about CD4⁺/CD8⁺ ratio in the lung tissue of NSIP in previous studies. We believe that insight into the features of T lymphocyte subsets distribution in different anatomical regions would help us understand the inflammatory process of NSIP.

**CD4⁺/CD8⁺ ratio of BALF predicts the pathological pattern of NSIP**

Over the past three decades, the technique of bronchoalveolar lavage has yielded important information in staging the pathological pattern of NSIP and predicting response to therapy. Nagai’s work about the relationship between lymphocyte subsets in BALF and clinical outcomes showed that low CD4⁺/CD8⁺ ratio of BALF in cellular NSIP correlated with better prognosis, while patients with fibrotic NSIP and IPF had high CD4⁺/CD8⁺ ratio and worse prognosis. Yamadori found that in NSIP patients with clinically evident pulmonary fibrosis, CD8⁺ TLs counts in BALF were reduced sharply. In present study, we analyzed T lymphocyte subsets in cellular and mixed/fibrotic patterns and compared the CD4⁺/CD8⁺ ratio in the various regions of lung tissue among the different pathological patterns. Our results showed that the changes of CD4⁺/CD8⁺ ratio in the lung tissue could be reflected through the CD4⁺/CD8⁺ ratio in BALF, which was correlated to the ratio in interstitial or small airway regions. Our observations that in NSIP patients the T lymphocyte subsets (CD8⁺ TLs) as well as the CD4⁺/CD8⁺ ratio recovered by BAL were related to those in lung tissue indicated that the use of a far less invasive technique of BAL, reliable information could be derived to predict the pathological patterns of NSIP.

We recognize that our study is limited by its retrospective analysis. B lymphocyte and other inflammatory cells are not investigated in this study. The second limitation was that the BALF was performed in middle lobe or lingula of the left upper lobe according ERS guideline for interstitial lung disease and the surgical biopsy in our study was performed in lower lobe, this may lead mismatch in T lymphocyte subsets between BALF and lung tissue. Nevertheless, to the best of our knowledge, no studies have evaluated T lymphocyte subsets in both surgically obtained lung tissue samples in different anatomical regions and BALF by quantitative immunohistochemistry and flow cytometry, respectively, in NSIP patients. We believe that the valid data in this study may reveal the pathogenic characteristics of NSIP.

Conclusion: In NSIP patients, T lymphocytes were distributed in different regions of lung tissues. CD8⁺ TLs
and CD4+/CD8+ ratios in BALF may reflect those in interstitial regions and around small airways.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.rmed.2012.09.021.

References


CD4+ and CD8+ T lymphocytes in lung tissue of NSIP

