Clinical Study

Increased Numbers of NK Cells, NKT-Like Cells, and NK Inhibitory Receptors in Peripheral Blood of Patients with Chronic Obstructive Pulmonary Disease

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most prevalent chronic adult diseases, affecting more than 200 million people worldwide, and has become the fourth leading cause of death [1]. The term includes a group of pulmonary disorders that cause dyspnea, which are characterized by structural changes in the lung and lead to progressive irreversible airflow limitation [2]. COPD is frequently associated with immune dysregulation, which makes the patients prone to infections. The number of CD4+ T lymphocytes and the CD4+/CD8+ ratio have been shown to be less in COPD patients compared with healthy volunteers [3–5], while the numbers of CD8+ T cells and B cells increase as COPD progresses [6–8].

In recent years, natural killer (NK) cells and natural killer T (NKT) cells have gained increasing attention. NK cells and NKT cells are the first line of defense against infection in the innate immune system, and they have the ability to directly kill target cells and interact with antigen-presenting cells as well as T cells [9–12]. NKT cells are innate-like T cells that constitute a minor lymphocyte population and exhibit features of both T cells and NK cells. Upon stimulation, NKT cells rapidly produce large amounts of cytokines [13]. Prieto et al. have revealed a functional defect in NK cells of patients with COPD; however, the number of NK cells does not differ significantly in peripheral blood between COPD patients and healthy controls [14]. Urbanowicz et al. have shown reduced NK cells and NKT cells in terms of both numbers and cytotoxicity in peripheral blood, while there
were not any differences in cytokine production in these cells [15]. Currently, the numbers and function of NK cells in COPD patients are controversial, and few in-depth studies have focused on the role of NKT cells in COPD.

NK cell function is determined by the integration of signals arising from the engagement of different NK receptors with specific ligands on potential target cells [16]. NK cell receptors can be divided into activating receptors (such as NKG2C, NKG2D, Nkp30, and Nkp46) and inhibitory receptors (such as CD158a, CD158b, KIR3DL1, and NKG2A) [17]. The presence of these receptors is not exclusive to NK cells, as NKT cells also express these similar receptors [18]. The inhibitory receptors discriminate healthy from diseased cells by surveying the surface expression of self-MHC class I molecules and prevent the attack of NK cells against these cells [19–22]. The activating receptors bind to host-derived or pathogen-encoded ligands, which are upregulated on stressed or infected cells. Once activated, NK cells and NKT cells can produce inflammatory cytokines, such as IFN-γ, and directly lyse target cells by exocytosis of perforin and granzyme [23]. However, little is known about NK cell and NKT cell receptors in COPD.

In order to verify the role of NK cells and NKT cells in COPD patients, we detected the numbers of CD3+CD56+ NK cells and CD3+CD56− NKT-like cells from the peripheral blood of COPD patients and compared their values with those in healthy nonsmokers (HNS) who acted as controls. We found that the numbers of CD3+CD56+ NK cells and CD3+CD56− NKT-like cells were greater, while the functions were impaired in COPD patients. The numbers of CD158a− and CD158b+ NK cells and CD158b+ NKT-like cells were greater in COPD patients, and the frequency of CD158b+ NK cells was negatively correlated with the forced expiratory volume in one second (FEV1) % prediction and the ratio of FEV1 to forced vital capacity (FVC).

2. Materials and Methods

2.1. Patients and Controls. Nineteen COPD patients were recruited from the outpatient clinic of the First Hospital of Jilin University from October 2011 to October 2012. Twelve HNSs from the medical examination center were recruited as controls. The patients were diagnosed as having COPD if their FEV1/FVC ratio was less than 70% and the FEV1 was predicted to be less than 80% after inhaling a bronchodilator. All 19 COPD patients inhaled salmeterol (50 μg twice daily) plus fluticasone propionate (500 μg twice daily) for long-term therapy. Participants were excluded if they had a history of tuberculosis, a clinical suspicion of current infection, or a history of physician-diagnosed asthma. COPD subjects were also excluded if they had an exacerbation within the previous 6 weeks, other pulmonary diseases, or severe functional defects of the heart, liver, or kidney. The patient characteristics are summarized in Table 1, and the lymphocyte subsets of the two groups were compared. The study was approved by the Human Ethics Committee of Jilin University, ChangChun, China. Written informed consent was obtained from each participant.

2.2. Pulmonary Function. Spirometry was performed using a dry rolling-seal spirometer (JAEGER Master Screen, USA). A standardized spirometry protocol, which exceeded the American Thoracic Society (ATS) testing standards, and a strict quality control program were used to obtain acceptable and reproducible data [24]. Values obtained were FEV1 % prediction and the FEV1/FVC ratio. The FEV1 % prediction and FEV1/FVC ratio should be more than 80% and 70% of the prediction, respectively, in normal pulmonary function.

2.3. Computed Tomography (CT) Scan. CT is more sensitive than plain radiographs, and it allows for a more detailed evaluation of the pulmonary parenchyma and surrounding structures. All patients underwent a CT scan. The COPD patients showed hyperinflation and enhancement of pulmonary markings.

2.4. Flow Cytometry of Lymphocyte Subsets. Human peripheral blood samples were stained with various monoclonal antibodies (mAbs) to determine the frequency of lymphocyte subsets in each individual using flow cytometry. The following mAbs and reagents were used in this study: fluorescein-isothiocyanate- (FITC-) conjugated anti-CD4 mAb, phycoerythrin- (PE-) conjugated anti-CD8 mAb, peridinin-chlorophyll-protein- (PerCP-) conjugated anti-CD3 mAb (Clone SK7/SK1/SK3, BD Tristest, San Jose, CA, USA), FITC-conjugated anti-CD3 mAb, and phycoerythrin- (PE-) conjugated anti-CD19 mAb (Clone SK7/4G7, BD Tristest, San Jose, CA, USA). Briefly, 100 μL of blood was

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HNS (n = 12)</th>
<th>COPD (n = 19)</th>
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<tbody>
<tr>
<td>Male/female</td>
<td>6/6</td>
<td>10/9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.75 ± 6.47</td>
<td>55.84 ± 5.72</td>
</tr>
<tr>
<td>Onset time (years)</td>
<td>0</td>
<td>20 ± 7.41</td>
</tr>
<tr>
<td>WBC count (×10³/L)</td>
<td>5.22 ± 1.02</td>
<td>6.04 ± 1.76</td>
</tr>
<tr>
<td>FEV1 (%)</td>
<td>88.28 ± 6.13</td>
<td>46.02 ± 14.20*</td>
</tr>
<tr>
<td>FEV1/FVC ratio</td>
<td>76.69 ± 2.69</td>
<td>55.3 ± 8.60*</td>
</tr>
</tbody>
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Hyperinflation/enhancement of pulmonary markings | 0 | 19 |

Data are shown as average ± SD; WBC: white blood cell count, FEV1: forced expiratory volume in one second, FVC: forced vital capacity. ∗P < 0.05 versus the HNS.
used for each staining experiment, and the samples were incubated for 30 min at room temperature. Erythrocytes were lysed using BD FACS lysis Solution 2 (BD Bioscience, San Diego, CA, USA). After washing with phosphate-buffered saline (PBS), the cells were subjected to flow cytometric analysis using a FACS Calibur (Beckton Dickinson) and FlowJo software (v5.7.2) (TreeStar, Ashland, OR, USA).

2.5. Flow Cytometry of NK Cells, NKT-Like Cells, and NK Receptors. Individual venous blood samples were subjected to flow cytometric analysis using specific antibodies. Briefly, 100 μL of blood samples was incubated in a mixture of FITC-conjugated anti-CD3, allophycocyanin- (-APC-) conjugated anti-CD56 (Clone B159, BD Pharmingen, San Diego, CA, USA), PerCP-conjugated anti-CD16 (Clone 3G8, BD Pharmingen, San Diego, CA, USA), and phycoerythrin-(PE-) conjugated anti-NKG2D (Clone I3D11, Biolegend, San Diego, CA, USA), anti-NKp30 (Clone p30-15, BD Bioscience, San Jose, CA, USA), anti-NKp46 (Clone 9E2/NKp 46, BD Bioscience, San Jose, CA, USA), anti-NKG2A (Clone I3H411, R&D Systems Inc., Minneapolis, MN, USA), anti-KIR3DL1 (Clone VL 186-16, Biolegend, San Diego, CA, USA), anti-CD158a (Clone HP-3E4, BD Bioscience, San Jose, CA, USA), and anti-CD158b (Clone CH-L, BD Bioscience, San Jose, CA, USA) for 30 min at room temperature, respectively. Mouse IgG1 (Clone MOPC-21, BD Pharmingen, San Diego, CA, USA) or control IgG2a (Clone G155-178, BD Pharmingen, San Diego, CA, USA) were used as negative controls. The remaining erythrocytes were lysed using BD FACS lysis Solution 2 (BD Bioscience, San Jose, CA, USA), and the frequencies of different subsets of NK cells were determined by flow cytometry using a FACS Calibur (BD Bioscience) and FlowJo software (v5.7.2).

2.6. The Detection of IFN-γ Secreted by NK and NKT-Like Cells. Peripheral blood mononuclear cells (PBMCs) were isolated from individual heparinized blood samples by density-gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Little Chalfont, UK). Next, PBMCs (10^6 cells/well) were incubated in complete RPMI 1640 culture medium in the presence of 4% paraformaldehyde, the frequencies of CD107a+ NK cells and CD107a+ NKT-like cells were determined by flow cytometric analysis using a FACScalibur; at least 10,000 events per sample were analyzed.

2.7. The Degranulation of NK and NKT-Like Cells. PBMCs were stained as described above. The PBMCs (10^6 cells/well) were cocultured in duplicate with K562 cells at a ratio of 10:1 of effector to target (E:T) in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) or control IgG2a (Clone GI55-178, BD Pharmingen, San Diego, CA, USA) for 6 h. The PBMCs cultured alone served as negative controls. Subsequently, the cells (10^6/tube) were stained in duplicate with FITC-anti-CD3 and APC-anti-CD56 at room temperature for 30 min, respectively. After washing with PBS (containing 1% fetal calf serum and 2.5% paraformaldehyde), the frequencies of CD107a+ CD3+ CD56+ NK cells and CD107a+ CD3+ CD56+ NKT-like cells were determined by flow cytometric analysis using a FACScalibur; least 10,000 events per sample were analyzed.

2.8. Statistical Analysis. Data are expressed as average ± standard deviation (SD) unless specified otherwise. The differences between the two groups were analyzed by the Wilcoxon rank sum test using SPSS 18.0 software. The relationship between two variables was evaluated using the Spearman rank correlation test. A two-sided P value < 0.05 was considered statistically significant.

3. Results

3.1. Imbalance of Immune Function in COPD Patients. To confirm the change of lymphocyte subsets in COPD patients, we detected the numbers of T cells, B cells, and NK cells from the peripheral blood of COPD patients by flow cytometry. Our research revealed that the numbers of CD3+ CD56+ NK cells and CD3+ CD56+ NKT-like cells were greater (P = 0.011, P = 0.037, resp.), while the numbers of CD3+ T cells, CD4+ T cells, and the CD4+/CD8+ ratio were less (P = 0.012, P < 0.001, P = 0.006, resp.) in COPD patients compared with HNS (Figures 1b, 1c, 1e, 1g). The number of CD8+ T cells was not statistically different in COPD patients compared with HNS (P = 0.491, Figure 1d). In addition, there was no significant difference in the numbers of B cells and CD3+ CD16+ NK cells between COPD patients and HNS (data not shown). Figure 1(a) shows the representative charts of CD3+ T cells, CD4+ T cells, CD8+ T cells, CD3+ CD56+ NK cells, and CD3+ CD56+ NKT-like cells in individual subjects from different groups.

3.2. Functionally Impaired NK Cells and NKT-Like Cells in COPD Patients. Our previous research indicated that the numbers of NK cells and NKT-like cells were greater in COPD patients compared to HNS. Subsequently, we studied the functions of NK cells and NKT-like cells by detecting the secretion of IFN-γ and CD107a degranulation in NK cells and NKT-like cells. As shown in Figures 2(b), 2(d), 2(f), and 2(h), there were no significant differences in the frequencies of CD107a+ NK cells and CD107a+ NKT-like cells.
stimulated by K562 cells between patients and controls, while the frequencies of IFN-γ-secreting NK cells and NKT-like cells stimulated by PMA and ionomycin were significantly less in COPD patients than in controls ($P = 0.001$, $P = 0.037$, Figures 2(a), 2(c), 2(e), and 2(g)).

3.3. Increased Numbers of CD158a+ NK Cells, CD158b+ NK Cells, and CD158b+ NKT-Like Cells in COPD Patients. As mentioned above, NK and NKT-like cell functions are associated with a balance of activating and inhibitory receptors [16]. Therefore, we analyzed the expression of NK and NKT-like cell receptors including NKG2C, NKG2D, NKP30, NKP46, CD158a, CD158b, KIR3DL1, and NKG2A. We found that the numbers of CD158a+ and CD158b+ NK cells as well as CD158b+ NKT-like cells were significantly greater in COPD patients compared with HNS ($P = 0.003$, $P < 0.001$, $P = 0.007$, resp.) (Figure 3). Differences between the other

Figure 1: Lymphocyte subsets in COPD patients and HNS. (a) The cells were gated initially on living lymphocytes. At least 50,000 events were analyzed for each sample. Viable lymphocytes were gated on the basis of forward and side angle light scattering characteristics. Data shown are representative charts from different groups of subjects and the frequencies of CD3+ T cells, CD4+ T cells, CD8+ T cells, NK cells, and NKT-like cells in individual subjects. (b), (c), (d), (e), and (f) Summarized data show the number of CD3+ T cells, CD3+CD4+ T cells, CD3+CD8+ T cells, CD3−CD56+ NK cells, and CD3+CD56+ NKT-like cells in COPD patients and HNS. (g) Summarized data show the CD4+/CD8+ ratio. The horizontal lines show the median.
receptors were not significantly different between COPD patients and HNS (data not shown).

3.4. The Frequency of CD158b+ NK Cells Was Negatively Correlated with Pulmonary Function in COPD Patients. Pulmonary function, especially FEV1/% prediction and the FEV1/FVC ratio, is often used to evaluate the severity of COPD. For this reason, we analyzed the correlation between pulmonary function and lymphocyte subsets. We found that there was no correlation between pulmonary function and the frequencies of CD3+ T cells, CD4+ T cells, CD8+ T cells, NK cells, CD158a+ NK cells, CD158b+ NKT-like cells, and B cells in COPD patients (data not shown). However, the frequency of CD158b+ NK cells was negatively correlated with the FEV1/% prediction and the FEV1/FVC ratio ($r = -0.473$, $P = 0.041$; $r = -0.649$, $P = 0.003$, resp.) (Figure 4).

4. Discussion

The results of the present study showed that the numbers of CD3+ T cells and CD4+ T cells and the CD4+/CD8+ ratio were significantly less in COPD patients compared with HNSs. These findings are similar to those reported previously [3–5]. However, the number of CD8+ T cells found in this study is different from other studies [7]. T cells can cause tissue injury either by direct cytolytic activities or through the secretion of proinflammatory mediators that recruit and activate other immune cell types [25]. CD4+ T cells are critical drivers of the B cell-dependent autoantibody response [26] and act as regulatory elements in the activation as well as the deletion of CD8+ T cells [5]. CD8+ T cells play a destructive role in COPD and can release proteolytic enzymes such as granzyme, which causes the death of structural cells [15, 27]. A decreased CD4+/CD8+ ratio is a characteristic feature of the pulmonary inflammatory response in COPD [27, 28], which is consistent with our results. Our data indicate that there is an imbalance of immune function in COPD patients, and this may explain why COPD patients are more prone to infections. B cells are the most important effector cells of humoral immunity. Active B cells recognize the antigen, develop into plasma cells that secrete immunoglobulins, and are finally converted to memory cells. Active T cells secrete cytokines, such as IL-2, IL-4, and IL-5, and act on B lymphocytes to induce proliferation [26]. In the present study, the number of B cells was not significantly different between COPD patients and HNSs. Moreover, no correlation between B cells and pulmonary function was found.

NK cells are the first line of defense against infection in the innate immune system because they can be activated by cytokines [29]. When activated, NK cells will directly...
kill target cells through the release of cytolytic granules by forming pores in the membrane of target cells [30]. NKT cells exhibit features of both T cells and NK cells and contribute to combating intracellular pathogens [13]. Our data revealed that the numbers of CD3−CD56+ NK cells and CD3+CD56−NKT-like cells were significantly greater in COPD patients compared to HNS, indicating that CD3−CD56+ NK cells and CD3+CD56−NKT-like cells may be involved in the pathogenesis of COPD. However, these results are not consistent with previous findings [14, 15]. In homeostasis, the function of NK cells in bronchoalveolar lavage fluid or lung tissue is suppressed; although they can conjugate with target cells, their cytotoxic capacity is profoundly impaired [31, 32].

Once infection has developed, large numbers of NK cells are recruited into the lung, and then they are activated to secrete cytokines, mainly IFN-γ [33]. Subsequently, we studied the functions of NK cells and NKT-like cells by detecting the secretion of IFN-γ and CD107a degranulation in these cells. We found that the frequencies of IFN-γ-secreting NK cells and NKT-like cells were significantly less in COPD patients than in HNS. These data indicate that NK and NKT-like cell functions are impaired in COPD patients, which explains why COPD patients are more susceptible to infection.

NK and CD3+CD56−NKT-like cell functions are associated with a balance of activating and inhibitory receptors. Our data indicate that NK and CD3+CD56−NKT-like cell functions
functions are impaired in COPD patients; therefore, we analyzed the activating and inhibitory receptors of NK cells and CD3^+CD56^+ NKT-like cells. Finally, we showed conclusively that the inhibitory receptors CD158a and CD158b on NK cells and CD158b on NKT-like cells were significantly greater in COPD patients compared with HNS. No differences in the other receptors evaluated were observed. CD158b is a killer cell immunoglobulin-like receptor (KIR) that has specificity for HLA-C antigen [34]. CD158b contains D1 and D2 extracellular immunoglobulin-like domains and a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibition motifs (ITIMs) that recruit and activate SHP-1 and SHP-2 phosphatases for inhibitory signal transduction [35, 36]. CD158a, also an inhibitory receptor, is similar in structure and function with CD158b. Accordingly, we presume that impaired NK and CD3^+CD56^+ NKT-like cell functions may be associated with increased inhibitory receptors CD158a and CD158b.

The dysfunction of lymphocyte subsets was observed in COPD patients. To demonstrate whether this dysfunction correlated with the severity of COPD, we assessed the correlation between the frequency of lymphocyte subsets and pulmonary function. Different from other studies showing that COPD severity is significantly and inversely associated with the frequency of circulating CD4^+ T cells and the CD4^+/CD8^+ ratio [37], no such correlation was observed in our research. This difference may be due to the patients' respiratory conditions and matching the condition to pulmonary function determination. Through further correlation analysis between NK cell subsets and pulmonary function, we found that the frequency of CD158b^+ NK cells was negatively correlated with FEV₁ and the FEV₁/FVC ratio. No correlation was observed between the frequencies of CD158a^+ NK cells or CD158b^+ NKT-like cells and pulmonary function. Thus, our data indicate that the frequency of CD158b^+ NK cells reflects the severity of COPD to a certain extent.

5. Conclusion
In conclusion, our data indicate that there is an imbalance of immune function in COPD patients. Increased numbers of NK cells and NKT-like cells expressing inhibitory receptors may participate in the pathogenesis of COPD, and the frequency of CD158b^+ NK cells may reflect the severity of COPD to a certain extent.

Conflict of Interests
The authors state that they have no conflict of interests.

References


