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Increased expression of $CD4^+IL\text{-}17^+$ cells in the lung tissue of patients with stable chronic obstructive pulmonary disease (COPD) and smokers

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ABSTRACT

$CD4^+IL\text{-}17^+$ cells have an important role in controlling immune and inflammatory reactions. The authors of the present study hypothesize that these cells may be involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). To characterize the frequency of $CD4^+IL\text{-}17^+$ cells in the lung alveolar walls, small airways and muscular pulmonary arteries of nonsmokers, smokers with normal lung function and COPD patients, $CD4^+IL\text{-}17^+$ cell number was assessed using double immunofluorescence staining, and IL-17 and IL-21 expression were measured using real-time quantitative PCR in the peripheral lung tissues of 10 nonsmokers, 10 smokers with normal lung function and 10 smokers with stable COPD. In the lung alveolar walls, the number of $CD4^+IL\text{-}17^+$ cells was increased in COPD patients compared with nonsmokers and in normal smokers compared with nonsmokers. In the small airways, the $CD4^+IL\text{-}17^+$ cell numbers were higher in COPD patients than in normal smokers and nonsmokers. A positive correlation was observed between $CD4^+IL\text{-}17^+$ cell expression and pathological changes in the lung tissue. In the small airways, the number of $CD4^+IL\text{-}17^+$ cells was positively correlated with airflow limitations. The IL-17 mRNA levels in lung tissues were increased in COPD patients and normal smokers compared with nonsmokers. Increased $CD4^+IL\text{-}17^+$ cell number in lung tissue is involved in chronic inflammation of the lungs and parallels lung injury aggravation in COPD patients and in smokers without airway limitations. These findings contribute to a better understanding of $CD4^+$ cell-related pathogenesis in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease involving various immune cells, including macrophages, neutrophils and, particularly, T lymphocytes. Pathology studies show that in COPD, inflammation occurs in the central and peripheral airways (bronchioles) and lung parenchyma [1]. Previous studies have emphasized the potential role of many inflammatory cells, particularly T lymphocytes, in the pathogenesis of COPD [2–4]. It is generally accepted that $CD4^+$ cells play a crucial role in the pathogenesis of COPD. However, the precise involvement of $CD4^+$ cells has not been confirmed.

T helper 17 (Th17) cells are defined as $CD4^+$T-lymphocytes that predominantly secrete the cytokine IL-17 [5], which has been demonstrated to be a mediator of inflammation in various autoimmune diseases [6]. Retinoic orphan receptor gamma t (ROR gamma t), with its human homologue RORC2, is the transcription factor required for the development of Th17 cells [7]. Interleukin-17A (IL-17A) and IL-17F secreted by Th17 could promote neutrophil chemotaxis and stimulate mucin production by the respiratory epithelium in COPD pathogenesis [8,9]. In addition, IL-21 from Th17 is necessary to provide the positive feedback loop for the successful amplification of Th17 cells [10]. IL-21 could promote the differentiation of naive CD8 T cells to a unique effector phenotype, through providing a signal required by naive CD8 T cells to differentiate in response to Ag and costimulation [11]. IL-21 deficiency could influence CD8 T cell quality and recall responses following an acute viral infection, and in the absence of IL-21, the capacity of CD8 T cells to attain the polyfunctional trait of IL-2 production is consistently reduced during both the effector and memory phases [12].

The exact role of Th17 immune responses in the development of COPD remains unconfirmed. In a chronic smoke-induced emphysema mouse model, the number of $IL\text{-}17^+$ cells in bronchoalveolar lavage fluid (BALF) was increased [13]. In COPD patients, the frequency of Th17 cells in peripheral blood and $IL\text{-}17A^+$ cells in bronchial submucosa were upregulated compared with controls [14,15]; however, no differences were found between COPD patients and controls in the IL-17 levels in sputum [16]. These previous studies did not report the frequency and distribution of $CD4^+IL\text{-}17^+$ cells in the lung tissues of COPD patients or the influence of $CD4^+IL\text{-}17^+$ cells on the pathological lung injury and airway limitation.

In our previous work, we found an increased expression of ROR gamma t and $IL\text{-}17^+$ cells in the lung tissues of COPD patients compared

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with normal smokers and nonsmokers [17]. We also found that the expression of IL-17$^+$ cells in the lung tissue of COPD patients was positively correlated with the number of CD4$^+$ cells and the degree of pathological lung injury [18]. Eustace et al. demonstrated that IL-17 could be expressed in the lymphocytes in peripheral lung tissue from COPD patients, but those authors could not identify the subsets of the lymphocytes [19]. Based on these findings, we hypothesize that upregulated IL-17 can be expressed in CD4$^+$ cells and can play a role in the lung pathological injury of COPD patients. Thus, we investigated the frequency and distribution of CD4$^+$IL-17$^+$ cells in the peripheral lung tissues of COPD patients and smokers with normal lung function.

Furthermore, it has been widely accepted that the pattern of inflammation in smokers without airway limitation is similar to that of COPD patients; however, in COPD patients, this inflammation is amplified. The chronic inflammation in COPD lungs might begin without airway limitation in smokers. In smokers who develop COPD, T-lymphocytes play a crucial role in the development of this inflammation [20]. However, the role of CD4$^+$IL-17$^+$ cells in the development of COPD in normal smokers is unclear; furthermore, CD4$^+$IL-17$^+$ cells' role in the chronic lung-inflammation of COPD patients and smokers has not yet been investigated. Thus, it is important to investigate the expression of CD4$^+$IL-17$^+$ cells in the lung tissues of both smokers without airway limitation and COPD patients.

The aim of this study was to investigate the expression and distribution of CD4$^+$IL-17$^+$ cells in lung alveolar walls, small airways and pulmonary arteries and to determine its relationship with lung pathological changes and airway limitation in COPD patients and in smokers without airway limitation. The expression of CD4$^+$IL-17$^+$ cells in lung tissues was evaluated, and the CD4$^+$IL-17$^+$ cell-related cytokines IL-17 and IL-21 were also investigated.

2. Materials and methods

2.1. Study subjects

A total of 30 subjects undergoing lung resection for a solitary peripheral carcinoma were recruited (Table 1). The subjects were subdivided into three groups: nonsmokers with normal lung ventilation function, current smokers with normal lung ventilation function and smokers with stable COPD. COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [21]. Patients were diagnosed with COPD if they had a forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) ratio<70%, with a reversibility of less than 15% after inhaling 200 mg of salbutamol. In the month preceding the study, none of the study subjects had suffered a recent exacerbation, defined as increased dyspnea associated with a change in the quality and quantity of sputum that would have led them to seek medical attention. All subjects were free of acute upper respiratory tract infections, and none had received glucocorticoids, theophylline or antibiotics within the preceding month. All subjects were nonatopic, had negative skin tests for common allergen extracts and had no past history of asthma or allergic rhinitis.

Table 1
Subject characteristics.

Subject group	NS (n = 10)	S (n = 10)	COPD (n = 10)
Age (years)	61 ± 9	63 ± 9	62 ± 9
FEV1%pred	102 ± 13	93 ± 8	55 ± 18[bd]
FVC% pred	98 ± 12	91 ± 6	74 ± 18[bd]
FEV1/FVC%	96 ± 12	86 ± 8[a]	59 ± 9[bd]
RV% pred	63 ± 13	77 ± 14	104 ± 29[bd]
TLC% pred	60 ± 10	68 ± 12	99 ± 28[bc]
RV/TLC	30 ± 4	34 ± 1[a]	55 ± 7[bd]
Male/female, n	8/2	9/1	10/0
Smoking history, pack-years	–	55 ± 18	63 ± 21
BMI kg/m^2	22.3 ± 3.3	21.6 ± 2.5	22.3 ± 2.3
6MWD m	462.42 ± 75.81	428.73 ± 58.46	373.37 ± 53.57[ac]
BODE	0(0,1)	1(0,1)	2(0,5)[ac]
MRC	0(0,1)	0(0,1)	1(0,2)[ac]
SGRQ	10(3,20)	25(1,32)	32(12,80)[bc]

Data are presented as the mean ± SD or median (range). COPD patients had not received any treatment within the preceding month. NS: nonsmokers; S: smokers with normal lung ventilation function; COPD: COPD patients; FEV1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; BMI: body mass index; 6MWD: 6-minute walking distance; BODE: body mass index, airflow obstruction, dyspnea and exercise capacity index; MRC: Medical Research Council scale; SGRQ: St. George Respiratory Questionnaire. a: P<0.05 and b: P<0.01, significantly different from nonsmokers; c: P<0.05 and d: P<0.01, significantly different from smokers.

The study was approved by a local ethical committee (the First Affiliated Hospital of Guangxi Medical University Ethical Committee, Guangxi, China) and conforms to the Declaration of Helsinki. Informed consent was obtained from all subjects.

The lung tissue specimens were obtained at surgery from the subpleural parenchyma of the lobe as far away as possible from the tumor site. Samples for histology and double immunofluorescence staining were transferred to 10% neutral buffer, fixed in formalin and embedded in paraffin. Samples for real-time quantitative polymerase chain reaction (RT-QPCR) were washed in normal saline, frozen within 15 min in isopentane precooled in liquid nitrogen and stored at −80 °C.

2.2. Pulmonary function tests

Pulmonary function testing was performed on a Jaeger MasterScreen spirograph (Jaeger Gmbh, Hoechberg, Germany) according to the Chinese Edition of National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology-Non-Small-Cell Lung Cancer Guideline 2006 during the week before surgery [22].

2.3. Histology and double immunofluorescence staining

The lung specimens were fixed, processed and embedded according to routine procedures. Deparaffinized sections (3 μm) were stained according to routine procedures with hematoxylin and eosin (HE) and were analyzed using light microscopy. For double immunofluorescence staining, formalin-fixed, paraffin-embedded tissue was cut into 3-μm-thick sections. Antigens were retrieved via high temperature treatment and pressure cooker heating for 90 s in citrate buffer (pH 6.0). The samples were then blocked with normal goat serum and incubated in monoclonal mouse anti-human CD4 (dilution 1:50, ab51312; Abcam Inc., Cambridge, MA, USA) and polyclonal rabbit anti-human IL-17A (dilution 1:400, sc-7927; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. The slides were then incubated with Cy3-conjugated goat anti-mouse IgG (dilution 1:50; Invitrogen, Paisley, UK) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:250, sc-3839; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 10 min at 37 °C and rinsed in PBS three times for 5 min.

2.4. Image analysis

The number of CD4$^+$IL-17$^+$ cells was calculated in the lung alveolar walls, small airways and pulmonary arteries. To obtain dual-label images, bright field and fluorescent images from the same field were captured and digitally merged to determine the CD4$^+$IL-17$^+$ cells. CD4$^+$IL-17$^+$ double-positive cells were analyzed at a magnification of 200×. Micrographs were obtained using a Leica TCS SP5 microscope (Leica Microsystems, Wetzlar, Germany) equipped with Image-Pro Plus V6.0 (Silver Spring, MD, USA). Adobe Photoshop 8.0 was used where applicable. The cases were coded, and the measurements were made by two pathologists who were blinded to the clinical data for the patient specimens. Disagreements were settled by consensus or referred to a third reviewer for adjudication.