Original Article

Peripheral Tc17 and Tc17/Interferon-γ Cells are Increased and Associated with Lung Function in Patients with Chronic Obstructive Pulmonary Disease

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is characterized by progressive loss of lung function and local and systemic inflammation, in which $CD8^+$ T-cells are believed to play a key role. Activated $CD8^+$ T-cells differentiate into distinct subpopulations, including interferon-γ (IFN-γ)-producing Tc1 and interleukin (IL)-17-producing Tc17 cells. Recent evidence indicates that Tc17 cells exhibit considerable plasticity and may convert into IL-17/IFN-γ-double producing (Tc17/IFN-γ) cells when driven by inflammatory conditions. The aim of this study was to investigate the Tc17/IFN-γ subpopulation in peripheral blood of patients with COPD and to evaluate their potential roles in this disease.
Methods: Peripheral blood samples were collected from 15 never-smokers, 23 smokers with normal lung function, and 25 patients with COPD (Global Initiative for Chronic Obstructive Lung Disease 2–4). Proportions of the IL-17/IFN-γ-double expressing subpopulation were assessed using flow cytometry. Plasma concentrations of cytokines favoring Tc17/IFN-γ differentiation were measured by enzyme-linked immunosorbent assay.
Results: Patients with COPD had higher proportions of Tc17 cells and Tc17/IFN-γ cells in the peripheral blood than smokers and never-smokers. The plasticity of Tc17 cells was higher than that of Th17 cells. The percentages of Tc17 cells and Tc17/IFN-γ cells showed negative correlations with forced expiratory volume in 1 s % predicted value ($r = -0.418$, $P = 0.03$; $r = -0.596$, $P = 0.002$, respectively). The plasma concentrations of IL-6, transforming growth factor-β1, and IL-12 were significantly higher in patients with COPD compared with smokers and never-smokers.
Conclusions: Peripheral Tc17 cells are increased and more likely to convert to Tc17/IFN-γ cells in COPD, suggesting that Tc17 cell plasticity may be involved in persistent inflammation of the disease.

Key words: $CD8^+$ T-cells; Interferon-γ; Interleukin-17; Plasticity

Introduction

Chronic obstructive pulmonary disease (COPD) is associated with enhanced and chronic inflammatory responses of the lungs to tobacco smoking and other noxious particles or gasses.[1] The inflammation in COPD, both in the lungs and in the systemic circulation, plays critical roles in disease development and progression.[2,3]

$CD8^+$ T-cells are major players in the inflammation and lung destruction of COPD.[4] It is believed that $CD8^+$ T-cells induce lung inflammation and emphysema in COPD by the production of granzyme B, perforin, and many other injurious and pro-inflammatory mediators. Studies in COPD patients have demonstrated that $CD8^+$ T-cells are accumulated in lung parenchyma and airways and correlate with the degree of airway obstruction.[5-8]

Activated $CD8^+$ T-cells differentiate into distinct subpopulations, including interferon-γ (IFN-γ)-producing

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Tc1, interleukin (IL)-4 producing Tc2, and IL-17-producing Tc17 cells, defined by selected sets of cytokines and transcription factors production.[9-11] Studies have shown that most $CD8^+$ T-cells isolated from the lung parenchyma of COPD patients are IFN-γ-producing Tc1 cells, exhibiting greater cytotoxicity compared with Tc2 cells.[12,13] Compared to Tc1 cells, Tc17 cells exhibit strikingly suppressed cytotoxic activity by secreting low levels of the cytotoxic T lymphocytes markers: T-bet, IFN-γ, perforin, and granzyme B.[14] Tc17 cells are shown to share some phenotypical properties with Th17, including retinoic acid receptor-related orphan receptor γt, CCR6, and IL-23R, and express tumor necrosis factor-α (TNF-α), IL-21, and IL-22.[15,16] Recent studies, however, have revealed that Tc17 cells are increased in several autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis, psoriasis, and are implicated in the pathogenesis of these diseases.[17-19]

Tc17 cells possess a high plasticity and can convert to IL-17/IFN-γ-double producing cells (Tc17/IFN-γ cells) permitted by IL-12 signaling, with distinct properties from Tc1 lineage.[20] Tc17/IFN-γ cells are highly cytotoxic and exhibit strong antitumor activity *in vitro* and *in vivo*. Interestingly, this unique subpopulation of Tc17 was found implicated in various inflammatory conditions in both humans and mice.[20-26]

Previous studies reported that IL-17A and IL-17F expressions by $CD8^+$ T-cells were increased in the airways of COPD patients.[27,28] However, little is known about the frequency of circulating Tc17 cells, particularly Tc17/IFN-γ cells and their associations with disease progression in COPD. Given that COPD is a lung disease with significant extrapulmonary effects, exploring the differentiation of peripheral $CD8^+$ T-cells, particularly the newly recognized Tc17 cells, may provide revealing evidence for the understanding of the mechanisms underlying systemic inflammation of the disease. Therefore, we assessed the signature cytokine IL-17 and IFN-γ expressions by $CD8^+$ T lymphocyte in peripheral blood from patients with COPD and analyzed the difference in the plasticity between Tc17 cells and Th17 cells. The cytokines believed to favor Tc17/IFN-γ differentiation were measured in plasma from the study subjects. Our results revealed higher proportions of Tc17 cells and Tc17/IFN-γ cells in peripheral blood from COPD patients, which could be explained by increased concentrations of IL-6, transforming growth factor β1 (TGF-β1) and IL-12. Importantly, the percentages of Tc17 cells and Tc17/IFN-γ cells were correlated negatively with forced expiratory volume in 1 s (FEV_1) % predicted. These results indicate that more studies are warranted to reveal the potential involvement of Tc17/IFN-γ cells in the pathogenesis of COPD.

Methods

Study subjects

Twenty-five male patients with COPD, all current or former smokers, were recruited for the study in the Beijing Tongren Hospital, Capital Medical University, China. Twenty-three smokers and 15 never-smokers with normal lung function were also included. The diagnosis of COPD was made according to clinical symptoms, a history of tobacco smoking, and impaired pulmonary function (postbronchodilator FEV_1/forced vital capacity <70%), according to the diagnostic criteria of the Global Initiative for Chronic Obstructive Lung Disease (2013).[1] All subjects with COPD were clinically stable and had not suffered any exacerbations for ≥3 months prior to enrollment. Smokers with normal lung function (FEV_1 >80% predicted) had a smoking history of ≥20 pack-years. Individuals with asthma, restrictive lung diseases, lung surgery, other chronic systemic inflammatory diseases, such as RA, type 1 diabetes mellitus, and inflammatory bowel disease, were excluded. The demographic and baseline clinical characteristics of the study participants are summarized in Table 1.

The study was approved by the local research ethics committee (TRECKT 2008-14). Written informed consent was obtained from all subjects.

Cell collection and flow cytometry

Peripheral blood samples were collected into ethylenediaminetetraacetic acid-treated tubes by venipuncture from the subjects after an 8-h fasting and were layered on the Ficoll-Paque Plus solution (Amersham Biosciences, Amersham, Bucks, UK) in a centrifuge tube, centrifuged at 400 × *g* for 20 min at 21°C, and peripheral blood mononuclear cells (PBMCs) were harvested. Then, divalent cation-free Hanks balanced salt solution was used for washing of cells at 300 × *g* for 5 min at 4°C. PBMCs were resuspended at 10^6 cells/ml in RPMI-1640 medium and prepared for the following procedures.

Freshly processed human PBMCs were stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate and 500 ng/ml of ionomycin in the presence of 5 μg/ml brefeldin A for 5 h

Table 1: The demographic and clinical characteristics of all participants

Items	Healthy nonsmokers	Smokers	Patients with COPD
Number of subjects	15	23	25
Age (years)	67.3 ± 6.5	66.4 ± 8.2	67.9 ± 7.7
Male/female (*n*/*n*)	15/0	23/0	25/0
Current/ex-smokers (*n*/*n*)	0	16/7	10/15
Pack-years, median (IQR)	0	39 (28–50)	46 (30–72)
FEV_1% predicted, mean ± SD	95.8 ± 6.2	91.3 ± 8.7	51.7 ± 15.5
FEV_1/FVC%, mean ± SD	83.2 ± 3.4	80.8 ± 4.9	55.6 ± 11.0
ICS use (*n*)	0	0	19
Bronchodilator use (*n*)	0	0	20
Exacerbations/year, mean ± SD	0	0	1.1 ± 0.3

Values are presented median (IQR) for smoking history, mean, and standard deviation for all others. COPD: Chronic obstructive pulmonary disease; FEV_1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; ICS: Inhaled corticosteroids; IQR: Interquartile range; SD: Standard deviation.

at 37°C as described by others.[29] The cells were harvested and stained with anti-hCD4-PE (BD Biosciences, San Jose, California, USA) and anti-hCD8-Percp (BD Biosciences) for 30 min at room temperature, followed by staining with anti-hIL-17A-FITC (eBioscience, San Diego, California, USA) and固定和透化后，抗hifn-γ−APC（EBISOSCIENCE）。 CD8⁺亚群是使用FACS -Calibur（BD Biosciences）确定的。总共为每个主题收集了总共1个× 10⁵事件，并通过FlowJo软件（Tree Star，Ashland，OR，USA）分析数据。

细胞因子酶连接的免疫吸附测定法

根据制造商的建议，通过酶联免疫吸附测定法（ELISA，Ebioscience，CA，CA，CA，美国，CA，CA，CA，CA，USA）的建议，根据制造商的建议，通过具有2 pg/ml，2.1 pg/Ml，2.1 pg/ML，ML，ML，ML，ML，ML，ML，ML，2.1 pg/6，通过酶联免疫吸附测定法（ELISA，CA，CA，美国，CA，CA，CA，CA，CA，CA，CA，CA，CA）测量IL -6，IL -12和TGF -β1的浓度。

统计分析

在适当的情况下，将组数据描述为平均值或中位数和四分位间范围的平均值和标准误差。使用单向方差分析（ANOVA）对正态分布进行了三组的比较，当检测到统计显着性时，两组之间的*post hoc*分析是通过使用Tukey测试执行。使用Pearson的等级相关系数分析了相关性。 *P*值< 0.05被认为具有统计学意义。所有分析均由Prism 5.02（GraphPad，La Jolla，CA，USA，美国）和SPSS进行的Windows Standard版本（SPSS Inc，美国芝加哥，伊利诺伊州）发行。

结果

慢性阻塞性肺部疾病患者中TC1细胞和TC17细胞的频率增加

我们首先使用流式细胞仪研究了研究受试者的外周血中的IFN-γ的频率。与吸烟者（中位数为56.60%， $P < 0.05$）相比，COPD患者（中位数为68.50%）的循环CD8⁺T细胞的TC1细胞比例更高。 Never -smokers [图1A和1C]。 与吸烟者（中位数为0.434%， $P < 0.01$）相比，COPD患者（中位数为0.562%）的患者（中位数为0.562%）患者的总循环CD8⁺T淋巴细胞的TC17细胞百分比增加了。

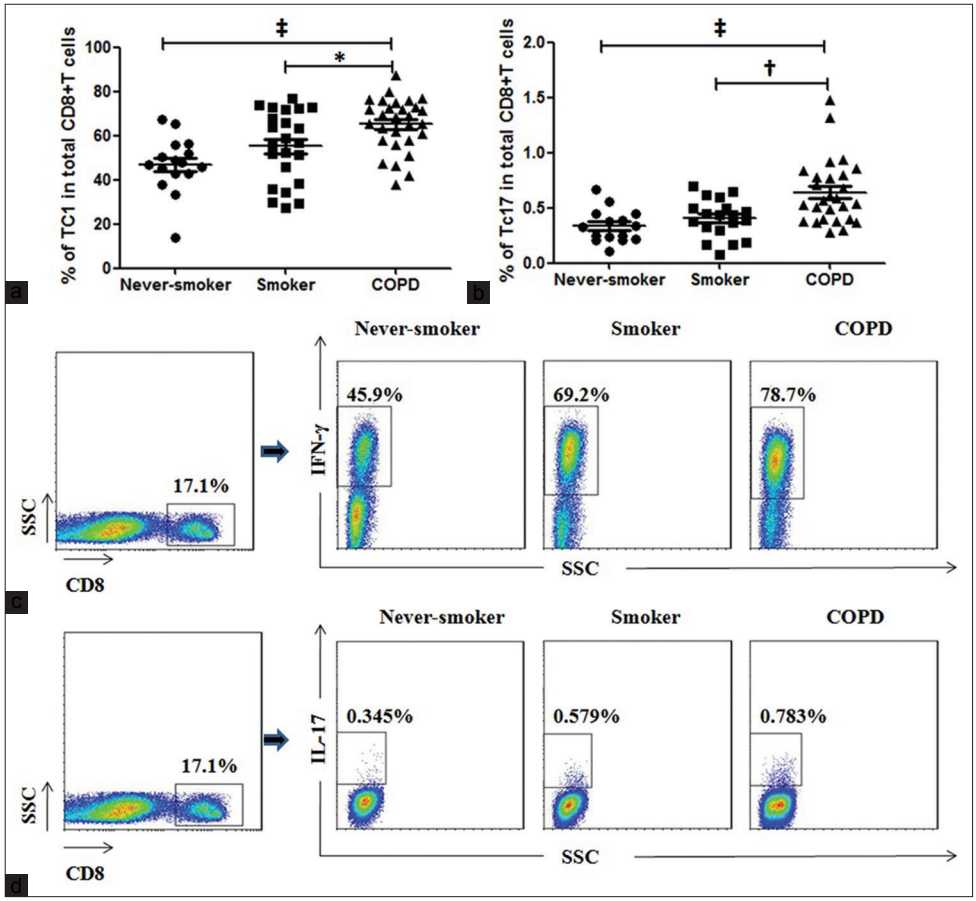

图1：来自慢性阻塞性肺部疾病，吸烟者和从未吸血的患者的外周血中的CD8⁺T细胞亚群。 分析了CD8⁺细胞的产生干扰素γ或白介素−17。 （a和b）来自慢性阻塞性肺部疾病，吸烟者和永无止境的患者的外周血中CD8⁺T细胞中TC1和TC17细胞的百分比。 （C和D）TC1和TC17细胞的代表性流式细胞仪。水平线表示中值。 SSC：侧面散射。 COPD：慢性阻塞性肺部疾病。 *$P < 0.05$，†$P < 0.01$，‡$P < 0.001$。

慢性阻塞性肺部疾病患者的双阳性TC17/Interferon - γ细胞的频率增加

在COPD的患者中，与吸烟者相比，周围血液中CD8 + T -cells（中位数为0.268%）中TC17/IFN- γ细胞的百分比明显更高。 0.001）[图2A和2C]。此外，在COPD患者中，观察到TC17细胞中TC17/IFN- γ细胞的百分比明显更高[中位数为48.09%；图2b]与吸烟者相比[中位数，31.25%， $P < 0.001$；图2b]和Never -Mmoker [中位数，26.67%， $P < 0.001$；图2b]，这表明在COPD中，TC17细胞与TC17/IFN- γ细胞的分化增加。

TC17细胞的可塑性高于慢性阻塞性肺疾病患者中Th17细胞的可塑性

如前所述，类似于Th17细胞，TC17表型不稳定。 TC17细胞可塑性（转换为TC17/IFN- γ细胞），由炎症环境驱动，尤其是由IL - 12，[22,28]高于Th17可塑性。[10]高于Th17的可塑性。 如图3B所示，TC17细胞中TC17/IFN- γ细胞（中位数为48.09%）的频率显着高于Th17细胞中Th17细胞中Th17/Th1细胞的频率（中位数，15.44%，$P < 0.001$）

在COPD患者中，表明TC17可塑性大于COPD中的Th17可塑性。 与以前的研究一致[10]在吸烟者和从未吸烟者中都可以看到相似的结果（数据未显示）。

双阳性TC17/Interferon- γ细胞的表达增加与1 s的强迫呼气量成反比

来自COPD患者的外周血中CD8 + T细胞中TC17细胞的百分比增加与FEV_1%预测值[$r = - - 0.418$， $P = P = 0.03$；图4a]。 更重要的是，COPD患者的CD8 + T −细胞中TC17/IFN- γ细胞的较高频率也与FEV_1%预测值[$r = - - - 0.596$，V29} 02;图4b]。

慢性阻塞性肺疾病中的白介素6，变化生长因子的浓度，转化生长因子β 1和白介素12增加

接下来，我们检查了据信驱动TC17/IFN- γ细胞分化的血浆细胞因子的浓度。 与吸烟者和从未吸烟者[$P < 0.01$，图5A - 5C]相比，COPD患者的血浆中IL - 6，TGF − β 1和IL - 12的浓度明显更高。这些表明，COPD中的TC17可塑性可能是由疾病的炎症环境驱动的。

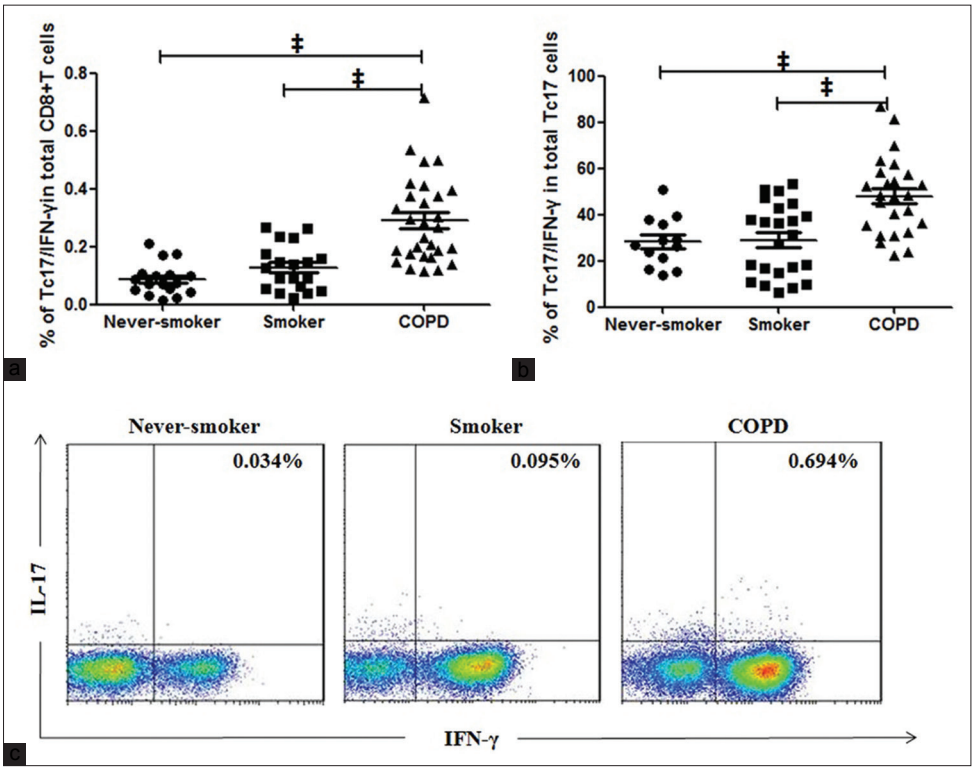

图2：患有慢性阻塞性肺部疾病，吸烟者和从未吸烟的患者的外周血中TC17/干扰素γ细胞亚群的比例。分析CD8 +细胞的生产白介素−17和干扰素− γ细胞在用Phorbol刺激5小时12-羟基苯二醇酯13-乙酸盐/离子霉素和Golgistop后5小时。 （a和b）来自慢性阻塞性肺部疾病，吸烟者和从不熟练的患者的CD8 + T -Cells和TC17细胞中TC17/干扰素γ细胞的百分比。 （c）TC17/干扰素γ细胞的代表性流式细胞仪。 COPD：慢性阻塞性肺部疾病。水平线表示中值值，‡$P < 0.001$。

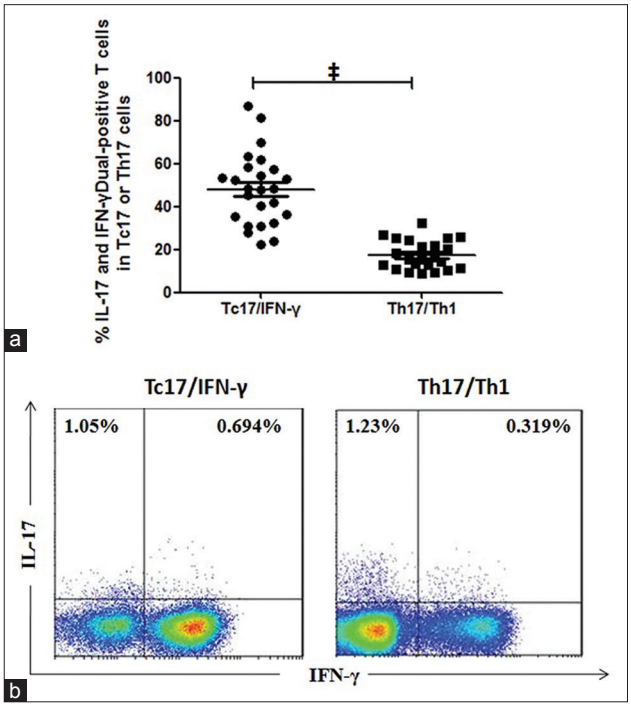

Figure 3: Frequencies of interleukin-17/interferon-γ-double positive subpopulations among Tc17 cells and Th17 cells in peripheral blood from patients with chronic obstructive pulmonary disease. $CD4^+$ cells and $CD8^+$ cells were analyzed for production of interleukin-17 and interferon-γ after 5 h of stimulation with phorbol 12-myristate 13-acetate/ionomycin and GolgiStop. (a) The percentages of Th17/Th1 cells and Tc17/interferon-γ cells among $CD4^+$ T-cells and $CD8^+$ T-cells in peripheral blood from patients with chronic obstructive pulmonary disease, respectively. (b) Representative flow cytometry of Th17/Th1 and Tc17/interferon-γ cells. Horizontal lines indicate median values, $^‡P < 0.001$.

Discussion

$CD8^+$ T-cells have long been recognized as the major pathogenic T-cells in airway inflammation and lung destruction in COPD. In contrast to the plenty of evidence supporting the accumulation and activation of $CD8^+$ T-cells in the lungs of COPD, little is known about the characteristics and functions of circulating $CD8^+$ T-cells in COPD, and the limited findings are often controversial. Domagała-Kulawik *et al*. reported that frequencies of circulating $CD8^+$ T-cells were increased in COPD while others found no differences in COPD patients compared with healthy subjects.[30-32] While these discrepancies might be due to the heterogeneous nature of the disease and different patient populations enrolled, however, functional plasticity and phenotype heterogeneity of $CD8^+$ T-cells are likely to be implicated.

Following the identification of an IL-17-producing subset within $CD8^+$ T-cells, studies have demonstrated that the so-called Tc17 cells are involved in a wide spectrum of immune diseases.[17-19,21,23-28] More recent evidence confirmed that the Tc17 lineage possessed a late developmental plasticity, that is, converting to Tc17/IFN-γ cells, in response to inflammatory signals.[20,22,26,33] As COPD is a lung disease with significant systemic inflammation, it is likely that circulating $CD8^+$ T-cells and/or their subpopulations are implicated in these pathogenic processes. We therefore hypothesized that Tc17 cells in COPD might show high plasticity and differentiate more to Tc17/IFN-γ cells, which, unlike Tc17 cells in general, are highly toxic, a property similar to Tc1 cells. We demonstrated here that the proportions of Tc17 cells, and more importantly, the multifunctional subpopulation Tc17/IFN-γ cells, were significantly increased in the peripheral blood of patients with COPD, and both were correlated negatively with FEV_1, a hallmark of severity of the disease.

Our findings of a higher proportion of Tc1 cells in peripheral blood from COPD patients compared with smokers and never-smokers are consistent with previous reports.[31,34] But our result of increased percentage of Tc17 cells in peripheral blood from patients with COPD was different from the study by Paats *et al*., who had found that the proportion of IL-17A positive $CD8^+$ T-cells was negligible in the peripheral blood, and no difference existed between COPD patients and healthy controls.[31] This discrepancy may be due to differences in disease severity (our patients had a higher mean FEV_1) and gender of the patients (our patients were all males). Moreover, we showed that the elevated frequency of circulating Tc17 cells was correlated significantly with COPD severity, suggesting that these cells may be involved in the pathogenesis of COPD. This is supported by animal studies demonstrating that the number of Tc17 cells was significantly increased in lungs of cigarette smoke-exposed mice, even after smoking cessation, and was correlated with lung emphysematous lesions.[31,35,36]

As mentioned earlier, Tc17 cells are far less cytotoxic as compared to Tc1 cells, and then by what mechanisms they may be pathogenic in COPD? As a novel subset of Tc17 cells, Tc17/IFN-γ cells are capable of acquiring strong cytotoxic function similar to Tc1 cells and expressing pro-inflammatory cytokines similar to their Th17/Th1 counterparts and therefore are believed to augment the pathogenic capability of Tc17 cells and promote exacerbation of a variety of autoimmune diseases.[20,22,24-26,37-40] Saxena *et al*. revealed that Tc17/IFN-γ cells might be indispensable for the aggravation of diabetes by direct cytotoxicity on the β-islet cells and expressing pro-inflammatory cytokines apart from IFN-γ in an experimental model of autoimmune diabetes.[24] Tajima *et al*. reported that Tc17/IFN-γ cells were rapidly generated in mesenteric lymph nodes, and IL-17 acted synergistically with IFN-γ to recruit effector $CD8^+$ T-cells and other inflammatory cells to colon tissues in a colitis model.[25] Here, we demonstrated for the first time to our knowledge that the percentages of Tc17/IFN-γ cells among $CD8^+$ T cells and Tc17 cells were significantly higher in peripheral blood from patients with COPD and correlated with FEV_1, suggesting that circulating Tc17/IFN-γ cells might be involved in persistent inflammation and loss of lung function in COPD. In addition, we found that Tc17 cells exhibited higher developmental plasticity than

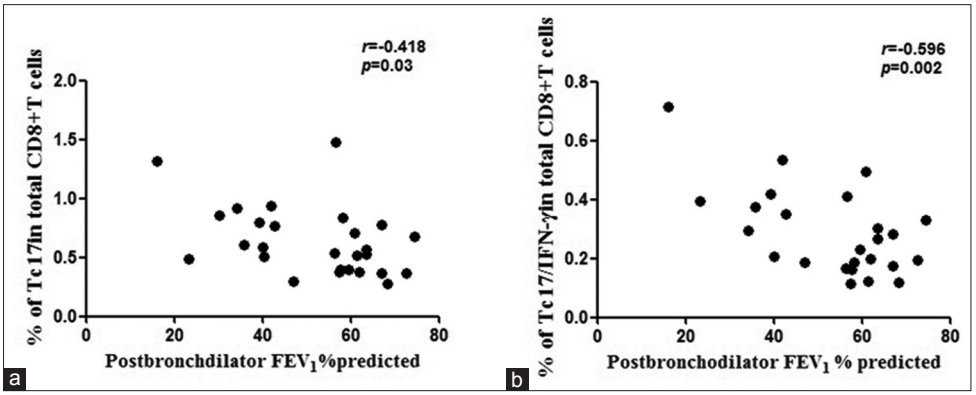

Figure 4: Correlations of Tc17/interferon-γ cells and lung function (n=25). (a) Frequencies of Tc17 cells in CD8$^+$ T-cells and (b) frequencies of Tc17/interferon-γ cells in CD8$^+$ T-cells correlated with forced expiratory volume in 1 s (FEV1)% predicted values in patients with chronic obstructive pulmonary disease. A P value < 0.05 was considered statistically significant.

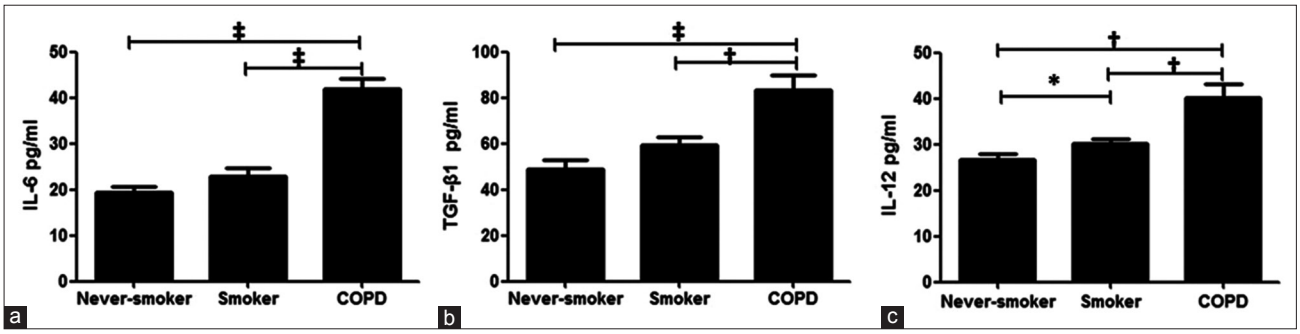

Figure 5: The concentrations of plasma cytokines. Interleukin-6 (a), transforming growth factor-β1 (b), and interleukin-12 (c) from patients with chronic obstructive pulmonary disease, smokers, and never-smokers. COPD: Chronic obstructive pulmonary disease. Horizontal lines indicate median values, *P < 0.05, †P < 0.01, ‡P < 0.001.

Th17 cells, although the implications of Tc17 plasticity in the pathogenesis of COPD remain speculative.

Since Tc17 plasticity is driven by inflammatory conditions, we supposed that the higher Tc17 plasticity was related to the systemic inflammation in COPD. Previous studies showed that in the presence of IL-6 and TGF-β1, human naïve CD8$^+$ T-cells acquired the Tc17 phenotype, and IL-12 was able to permit Tc17 cells to acquire the potential to produce IFN-γ, thus differentiating to Tc17/IFN-γ cells.[20,29,33] In the current study, we found increased concentrations of IL-6 and TGF-β1 in the plasma from patients of COPD. More importantly, consistent with other studies, the concentration of IL-12 was higher in patients with COPD as compared to the controls.[41,42] These results indicate that further studies are needed to explore the mechanisms by which these cytokines induce Tc17 plasticity in COPD.

Our study has several limitations. First, we did not examine the frequency of Tc17 and Tc17/IFN-γ cells in the lungs, for example, in bronchoalveolar lavage, which may be more relevant to airway diseases of COPD; hence, further investigations to this issue are needed. Second, we have only shown increased plasma levels of IL-6, TGF-β1, and IL-12 in COPD; whether and by what mechanism these cytokines promote Tc17 plasticity is still speculative. Third, some of our patients had used inhaled corticosteroids, and therefore the possibility of an effect of this medication on our results cannot be excluded, although Paats *et al*. found no effect of inhaled corticosteroids on peripheral CD8$^+$ T-cells in COPD.[31]

In summary, this study provides a comprehensive analysis of circulating CD8$^+$ T-cells and their subpopulations in COPD, with a novel finding that the circulating Tc17/IFN-γ cells, in addition to Tc17 cells, are significantly increased and correlated to the severity of disease, suggesting that these cells may be involved in the pathogenesis of COPD. Further studies are needed to elucidate the underlying mechanisms of CD8$^+$ T-cell heterogeneity and Tc17 cell plasticity in COPD, which may shed new light on the understanding of local and systemic inflammation characteristic of the disease.

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Conflicts of interest

There are no conflicts of interest.

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