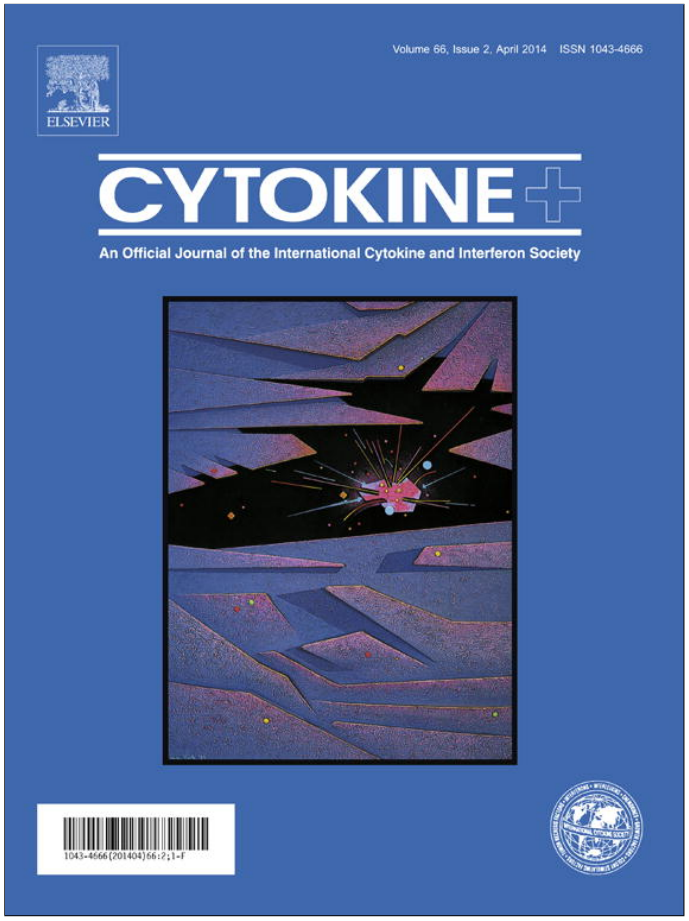
Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/authorsrights

Author's personal copy

Cytokine 66 (2014) 119–126

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients ☆

Domenico Galati [a], Marina De Martino [b], Annamaria Trotta [c], Gaetano Rea [d], Dario Bruzzese [e], Gaetano Cicchitto [f], Anna Agnese Stanziola [b], Maria Napolitano [c], Alessandro Sanduzzi [b], Marialuisa Bocchino [b,*]

[a] Dipartimento di Ematologia, IRCCS INT Fondazione Pascale, Napoli, Italy
[b] Dipartimento di Medicina Clinica e Chirurgia, Università Federico II, Napoli, Italy
[c] UOSC Immunologia Oncologica, IRCCS INT Fondazione Pascale, Napoli, Italy
[d] Dipartimento dei Servizi Diagnostici e Generali, Ospedali dei Colli, Monaldi-Cotugno-CTO, Napoli, Italy
[e] Dipartimento di Sanità Pubblica, Università Federico II, Napoli, Italy
[f] Fisiopatologia Respiratoria, ASL, Salerno, Italy

ARTICLE INFO

Article history:
Received 5 June 2013
Received in revised form 31 October 2013
Accepted 5 December 2013
Available online 10 January 2014

Keywords:
IPF
Flow cytometry
Th17 cells
Regulatory T cells
NK cells

ABSTRACT

The immune response plays an unsettled role in the pathogenesis of idiopathic pulmonary fibrosis (IPF), the contribution of inflammation being controversial as well. Emerging novel T cell sub-populations including regulatory T lymphocytes (Treg) and interleukin (IL)-17 secreting T helper cells (Th17) may exert antithetical actions in this scenario. Phenotype and frequency of circulating immune cell subsets were assessed by multi-parametric flow cytometry in 29 clinically stable IPF patients and 17 healthy controls. The interplay between Treg lymphocytes expressing transforming growth factor (TGF)-β and Th17 cells was also investigated. Proportion and absolute number of natural killer (NK) cells were significantly reduced in IPF patients in comparison with controls ($p < 0.001$). Conversely, the proportion and absolute number of $CD3^+CD4^+CD25^{high}Foxp\text{-}3^+$ cells were significantly increased in IPF patients ($p = 0.000$). As in controls, almost the totality of cells (>90%) expressed TGF-β upon stimulation. Interestingly, the frequency of Th17 cells was significantly compromised in IPF patients ($p = 0.000$) leading to an increased TGF-β/IL-17 ratio (4.2 ± 2.3 vs 0.5 ± 0.3 in controls, $p = 0.000$). Depletion of NK and Th17 cells along with a not compromised Treg compartment delineate the existence of an "immune profile" that argue against the recent hypothesis of IPF as an autoimmune disease. Our findings along with the imbalance of the Treg/Th17 axis more closely suggest these immune perturbations to be similar to those observed in cancer. Clinical relevance, limitations and perspectives for future research are discussed.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and devastating fatal illness with a mean survival of less than 5–6 years from the onset of clinically relevant symptoms. Disease incidence is on the rise with estimates ranging from 6.8 to 16.3 cases per year in the United States [1]. IPF prognosis in a variable proportion of cases may be further worsened by lung cancer with prevalence estimates ranging from 4.8% to 48% in different studies and a cumulative incidence rate increasing over time [2]. Mechanisms underlying IPF pathogenesis are elusive and not yet clearly elucidated. IPF is actually considered as an epithelial/fibroblastic disorder resulting from an unabated continuation of the repair process in response to recurrent episodes of epithelial injury [3]. Histologically, the disease hallmark is represented by the accumulation of fibroblastic foci composed of proliferating and migrating fibroblasts and of differentiated myofibroblasts accounting for the deposition of extracellular matrix and alveolus disruption [4]. In agreement with previous reports, we have recently shown that oxidative stress may act as a key player in the pathogenesis of IPF [5]; conversely, the role of inflammation along with the involvement of the immune response still remains largely controversial.

Emerging novel T cell sub-populations including T regulatory (Treg) lymphocytes and effector interleukin (IL)-17 secreting T helper cells (Th17) may exert antithetical actions in this scenario. Notably, regulatory T lymphocytes include different cell subsets indispensable for the maintenance of immune homeostasis. As a

☆ This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

* Corresponding author. Address: Department of Clinical Medicine and Surgery, Respiratory Medicine Section, Federico II University, Via L. Bianchi, 5, 80131 Naples, Italy. Tel.: +39 081 706 2773; fax: +39 081 770 2457.
E-mail address: marialuisa.bocchino@unina.it (M. Bocchino).

1043-4666/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.cyto.2013.12.003

Author's personal copy

consequence, any dysregulation of such cells may have a significant impact in a wide array of human diseases, including cancer [6]. Indeed, exuberant Treg function has been shown to play an important role in cancer-mediated immune-suppression [7]. On the other side, Th17 cells mainly exert a pro-inflammatory role and actively participate in the pathogenesis of autoimmune diseases, allergy and transplantation rejection [8]. Conversely, Th17 impairment contributes to tumor growth and progression [9,10]. Therefore, the better understanding of these players is of relevant interest especially in the light of the evolving concepts that suggest the contribution of both cancer-related mechanisms and of autoimmune processes in the natural history of IPF [11,12]. To date, only few data addressing the role of Treg cells in IPF pathogenesis are actually available, at least in the human model. In this issue, Kotsianidis et al. have firstly described a global Treg impairment in IPF patients that was strongly correlated with disease severity [13]. In agreement with these observations, Shimizu et al. have further shown that interstitial Foxp3-positive lymphocytes were decreased in the lung of patients with usual interstitial pneumonia (UIP), and at a lesser extent in that of patients affected by fibrotic non-specific interstitial pneumonia (f-NSIP) [14]. These findings were correlated with the severity of fibrosis and were due to an imbalance of the CXCL12/CXCR4 axis. Conversely, to our knowledge, there are no studies addressing the involvement of Th17 cells specifically in the clinical context of IPF.

Aim of the present study was to analyze in a more comprehensive fashion the *ex vivo* distribution and phenotype of immune cell subsets, including regulatory T cells, by means of multi-parametric flow cytometry at the peripheral level in a cohort of patients affected by clinically stable IPF. The dynamic inter-relationship between Treg lymphocytes and Th17 cells was also investigated by addressing their functional ability to express two cytokines that are pivotal in the modulation of the immune response, that are transforming growth factor (TGF)-β and IL-17, respectively.

2. Materials and Methods

2.1. Study population

Twenty-nine consecutive Italian patients affected by stable IPF who attended the Respiratory Medicine Division of the Federico II University at the Monaldi Hospital in Naples, Italy, were enrolled in the study. Diagnostic preliminaries included clinical history, physical examination, routine blood tests, lung function testing, arterial blood gases analysis, high resolution computed tomography scan (HRCT) of the thorax and echocardiography. The 6 minute walk test (6MWT) and fiberoptic bronchoscopy were performed in selected cases. Diagnosis of IPF was made according to the recent American Thoracic Society/European Respiratory Society consensus statement [15]. Exclusion criteria were acute disease exacerbation (including viral and bacterial infections) and systemic administration of corticosteroids and/or immune-suppressive drugs at enrolment or in the previous 60 days. Seventeen age- and sex-matched healthy volunteers were recruited as control group. The local Ethics Committee approved the study and all participants provided written informed consent. Demographics and clinical characteristics of the study population are shown in Table 1. Lung function parameters are reported in Table 2.

2.2. Phenotypic analysis of peripheral immune cell subsets

Fresh whole peripheral blood samples were drawn into 0.2% EDTA-containing Vacutainer tubes. Four aliquots were incubated for 30 min at 4 °C in the dark with the following cocktails of monoclonal antibodies (MAbs) conjugated to different fluorochromes, as reported: (1) Foxp3-FITC, CD152-PE, CD45RO-PerCP-Cy5.5, CD25-PE-CY7, CD45RA-APC, CD4-APC-CY7; (2) CD62L-FITC, Foxp3-PE, CD184-PerCP-Cy5.5, CD25-PE-CY7, CD13-APC, CD4-APC-CY7; (3) CD158-FITC, CD16-PE, CD161-PerCP-Cy5.5, CD56- PE-CY7, CD8-APC; and (4) CD4-PE, CD3-PerCP-Cy5.5, CD20-APC, CD8-APC-CY7. Matched isotype MAbs were used as negative controls. All reagents were purchased from Becton Dickinson (BD, San Diego, CA, USA). Intracellular staining for Foxp3-Alexa Fluor 488 (clone 259D/C7) and Foxp3-PE (clone 259D/C7) was performed according to the manufacturer's instructions (BD Bioscences Pharmingen). After red cell lysis with ammonium chloride (BD FACS Lysing Solution BD Bioscences Pharmingen), 50.000 total events were acquired for each sample by means of a 6-color staining FACS Canto II (BD). Data analysis was performed with the FACS Diva software (BD). Selection of the lymphocytes containing gate was based on side/forward scatter. An additional aliquot of blood was used for white blood cells and lymphocytes counts determination per mm^3 via haemocytometer (XT-1800i Sysmex Europe, Norderstedt, Germany). Frequency of Foxp-3^+ regulatory T lymphocytes was calculated as percentage of $CD3^+CD4^+CD25^{high}$ cells. The absolute number per mm^3 of any cell subset was calculated as follows: percent of a given cell subset × total number of lymphocytes per $mm^3/100$.

2.3. Dynamics of regulatory T lymphocytes and Th17 cells

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by centrifugation on a Ficoll-Hypaque gradient (Ficoll-Paque™ Plus, GE Healthcare Bio-Sciences AB), washed and resuspended in RPMI 1640 (Lonza, Bioresearch) supplemented with 10% fetal calf serum (EuroClone Group), 2 mmol/l L-glutamine (Lonza, Bioresearch), 10 UI/ml penicillin and 10 μg/ml streptomycin (Lonza, Bioresearch). For single-cell analysis of TGF-β and IL-17 synthesis, respectively by regulatory and Th17 cell subsets, two different aliquots of 1×10^6 PBMCs/ml were cultured in 3 ml of complete medium and stimulated with 10 ng/ml PMA and 1 μg/ml Ionomycin (both purchased from Sigma Aldrich) for 18 hrs at 37 °C and 5% CO_2. During the last 12 hrs of culture 2 μl/ml monensin (Golgi Stop, BD) was added in one out of two culture plates in order to induce the intracellular accumulation of newly synthesized proteins. The untreated plate was used as positive control of secretion of targeted cytokines. Two aliquots of 1×10^6 -cells/ml of monesin-treated cells were then stained with the following cocktails of monoclonal Abs: (1) Foxp-3-FITC, TGF-β-PE, CD45RO-PerCP-Cy5.5, CD25-PerCP-CY7, CD45RA-APC, CD8-APC-CY7; (2) CD4-PE, CD3-PerCP-Cy5.5, CD8-PerCP-CY7, IL-17-APC. A single aliquot of 1×10^6/ml monesin-untreated cells was instead stained with the following monoclonal Abs: Foxp-3-FITC, TGF-β-PE, CD4-PerCP-Cy5.5, CD25-PerCP-CY7, IL-17-APC, CD8-APC-CY7. Surface staining was performed as previously described. Intracellular staining of Foxp-3, TGF-β and IL-17 was performed at 4 °C for 30 min in previously saponin/formaldehyde (Cytofix/Cytoperm buffer purchased from BD) treated samples. Matched isotype MAbs were used as negative controls. The lymphocytic gate was selected as previously reported and used to count 30.000 events. Frequency of TGF-β expressing Foxp-3^+ regulatory T lymphocytes was calculated as percentage of $CD8^-$ $CD25^{high}$ cells. Frequency of Th17 cells was expressed as proportion of $CD3^+CD4^+CD8^-$IL-17^+ cells. The TGF-β/IL-17 ratio was calculated as follows: frequency of TGF-β expressing Foxp-$3^+CD8^-CD25^{high}$ regulatory T lymphocytes/frequency of $CD3^+CD4^+CD8^-$IL-17^+ cells.

2.4. Statistical analysis

All statistical analyses were performed with the SPSS version 16.0 (SPSS, Chicago, IL) and with the statistical platform R version

Author's personal copy

Table 1
Demographics and main clinical features of IPF patients.

Parameter	IPF patients	Controls
Age (years)	64 ± 9.8 (1.9); 65 [59.9–68.1]	62 ± 3.6
Gender	21 M/8 F	12 M/5 F
Smoking history	20 Ex/9 never smokers	10 Smokers/7 never smokers
Pack/years	40.5 ± 35.5 (8.6); 35 [22.2–58.8]	31 ± 11 (3.5); 30 [23.4–39.2]
Risk factors	Recurrent respiratory infections (n = 6) Occupational exposure (n = 8)	–
Time of first diagnosis	2008 (n = 3), 2009 (n = 5), 2010 (n = 8), 2011 (n = 9), 2012 (n = 4)	–
Emphysema Pulmonary hypertension[a]	n = 6 n = 5	–
Co-morbidities	Gastro-esophageal reflux (n = 10) Ischemic heart disease (n = 6) Systemic hypertension (n = 8) Type II diabetes (n = 6) Other (n = 10)	–
Previous IPF therapies	No (n = 14), Yes (n = 15) Corticosteroids (n = 12) N-acethyl-cysteine (n = 7) Immune-suppressor agents (n = 2) Experimental trials (n = 1)	–
Long term O_2 supplementation	No (n = 16), Yes (n = 13)	–

Data are expressed as means ± SD (SEM); median [IQR_{25}–IQR_{75}].
[a] sPAP (systolic pulmonary arterial pressure) was estimated by means of echocardiography.

Table 2
Functional characteristics of IPF patients.

Parameter	IPF patients	Controls
paO_2 (mmHg) (21% FiO_2 at rest)	69.5 ± 13.6 (2.8); 71.6 [63.8–75.3]	–
% SpO_2 (21% FiO_2 at rest)	92.3 ± 5.7 (1.2); 95 [89.9–94.7]	97 ± 1.4 (0.4); 97 [96–98][a]
FVC (L)	3.1 ± 4.0 (0.9); 2.4 [1.3–4.9]	3.4 ± 0.7 (0.3); 3.3 [2.6–4.0]
FVC (% pred)	69.8 ± 18.7 (3.9); 69 [61.5–78.1]	98.4 ± 13 (4.9); 91 [86.2–110]
TLC (L)	3.5 ± 1.3 (0.3); 3.3 [2.7–4.2]	5.0 ± 0.9 (0.3); 4.9 [4.3–5.6]
TLC (% pred)	60.6 ± 18.7 (5); 61 [49.8–71.4]	85.6–38 (1.2); 85.5 [82.8–88.3]
$DLCO_{sb}$ (Hb-adjusted) (ml/min/mmHg)	9.3 ± 4.5 (1.0); 8.7 [7.1–11.5]	23.3 ± 6.1 (2.0); 23.3 [18.5–27.9]
$DLCO_{sb}$ (Hb-adjusted) (% pred)	41.9 ± 17.8 (4.1); 41 [33.2–50.6]	85.1 ± 4.17 (1.3); 84.5 [82.1–88]
DLCO/AV (ml/min/mmHg)	3.1 ± 0.9 (0.2); 3.1 [2.6–3.5]	4.9 ± 0.7 (0.3); 4.9 [4.2–5.4]
DLCO/AV (% pred)	75.2 ± 25.1 (5.8); 81 [63.1–87.3]	109 ± 19.2 (6.8); 114 [93–125]
6 MWT distance (m)	405 ± 139 (35); 390 [331–479]	–

Data are expressed as means ± SD (SEM); median [IQR_{25}–IQR_{75}].
Abbreviations: PaO_2 = arterial O_2 partial pressure; FiO_2 = fraction of inspired oxygen; SpO_2 = oxygen saturation; FVC = forced vital capacity; $DLCO_{sb}$ = single breath diffusing lung capacity of carbon monoxide; Hb = haemoglobin; AV = alveolar volume; TLC = total lung capacity; and 6 MWT = six-minute walk test.
[a] Oxygen saturation was evaluated by means of percutaneous pulse-oxymetry.

2.15 (R Development Core Team, 2102). Continuous variables are reported as mean ± standard deviation (SD) (standard error of the mean, SEM) and median [25th–75th percentile range]; categorical variables are reported as number of occurrences and percentages. For all study variables, comparison among controls and IPF subjects was based on the non-parametric Wilcoxon–Mann–Whitney exact test. Spearman correlation coefficient was computed to investigate the correlations between the different cell subsets. For all analyses, we used two-sided tests, with p values <0.05 denoting statistical significance. However, due to the large number of the variables examined, in order to control the family-wise error rate at level a = 0.05, the significance of the p values associated to the Mann–Whitney U statistics and to the Spearman correlation coefficients was further assessed by using the adaptive Bonferroni procedure [16].

3. Results

3.1. IPF patients display a significant depletion of peripheral NK cells

Ex vivo frequency distribution and quantification of peripheral blood immune cell subsets were realized by means of multi-parametric flow cytometry analysis in 29 clinically stable IPF patients and in 17 healthy volunteers. Main results are displayed in Table 3. As shown, no significant differences were recorded comparing the mean proportions and absolute numbers (data not shown) of both $CD4^+$ and $CD8^+$ T cells and of their ratio in the two study groups. Interestingly, in agreement with a previous report by Gilani et al. [17] showing that the co-stimulatory molecule CD28 was down-regulated on circulating $CD4^+$ T lymphocytes in poor prognosis IPF patients, the percentage of $CD28^+CD4^+$ T cells was slightly decreased in IPF patients. However, this finding was no more relevant after multiple test correction. No differences were reported when comparing their absolute number in the study groups (data not shown). As for $CD4^+$ and $CD8^+$ T cells, no differences in the distribution of $CD20^+$ B lymphocytes were observed between IPF and control subjects. Conversely, both the proportion and the total count of NK cells were markedly decreased in IPF patients as compared to controls. Similarly, a significant decrease of the percentage of NKT cells (but not of their absolute number) was also found in IPF patients in comparison with the control group; however, this finding was no more statistically relevant after multiple test correction.

Author's personal copy

3.2. Frequencies and absolute numbers of regulatory T cells are increased in IPF

Flow cytometry estimates of frequencies of circulating regulatory T lymphocyte subsets are reported in Table IV. As shown, the mean proportion and absolute number of $CD3^+CD4^+CD25^{high}$-Foxp-3^+ cells were significantly increased in IPF patients in comparison with control subjects, as also confirmed by multiple test correction. However, no differences were found when looking at the distribution of naïve ($CD45RA^+$) and effector ($CD45RO^+$) subsets, the majority of cells (>90%) expressing the CD45RO marker in both study groups. In order to further characterize the distribution of blood T regulatory cells the expression pattern of three additional surface molecules, such as CXCR4, CTLA-4 and CD62L, was investigated. As shown in Table 4, again no substantial differences were recorded in IPF cases in comparison with control subjects Fig. 1.

3.3. Impairment of Th17 cells leads to a significant imbalance of the TGF-β/IL-17 axis in IPF

To address the extent of the functional performance of regulatory T cells in IPF, measurement of intracellular TGF-β expression was assessed upon *in vitro* stimulation as described in Section 2. Concomitantly, the ability of $CD4^+$ T lymphocytes to produce IL-17 was also investigated. As shown in Fig. 2A, the mean proportion of regulatory T cells expressing TGF-β was significantly higher in IPF patients than in controls. Almost the totality of cells was recruited upon stimulation in the two study groups with the involvement of both naïve and effector cells (Fig. 2, panel B). However, the contribution to TGF-β expression by the latter cell subset was less impressive in IPF patients (Fig. 2, panel B). Representative counter plots for TGF-β expression by regulatory T lymphocytes in an healthy subject and in an IPF patient are respectively shown in panels C and D of Fig. 2. Interestingly, the mean frequencies of IL-17 expressing helper T lymphocytes was significantly compromised in IPF patients, as illustrated in Fig. 3 (panels A and B). Representative counter plots for IL-17 expression by helper T cells in an healthy subject and in an IPF patient are shown in panels C and D (Fig. 3). Expression of TGF-β and IL-17 was not detectable in monesin-untreated cell samples suggesting that both cytokines were secreted upon synthesis (data not shown). Finally, as reported in Fig. 4, the TGF-β/IL-17 ratio was dramatically increased in IPF patients in comparison with controls. This finding was inversely correlated in a significant manner with the percentage of Th17 cells (Spearman Rho: −0.81, $p < 0.001$; significant after multiple test correction).

3.4. Frequencies and dynamics of blood immune cell subsets are not associated with IPF severity

No correlations were found between the distribution of any circulating immune cell subset studied (either as percentage or absolute number) and IPF associated parameters of disease severity, previous therapy and smoking history. Similarly, perturbations of TGF-β and IL-17 expression were not correlated with any disease-related indicator. Follow-up data are available over a period of 20 ± 3.8 months. Six patients are died due to rapidly worsening of respiratory failure, 15 cases have experienced a progressive clinical and functional decline, while the remaining 8 patients still remain stable, according to the reference criteria [15]. No correlations between the immunological parameters analyzed and the clinical outcome (that is, died, deterioration, or stability) were found.

Table 3
Distribution of peripheral immune cell subsets in IPF patients.

Parameter	CTR ($n = 17$)	IPF ($n = 29$)	p[a]	Significance after multiple test correction
WBCs count/μl	7711 ± 1918 (465) 7500 [6725–8698]	8842 ± 1988 (398) 8800 [8021–9663]	ns	No
% Lymphocytes	33.8 ± 7.1 (1.7) 33.1 [30.2–37.5]	29.9 ± 7.8 (1.5) 29.1 [26.7–33.1]	ns	No
Number of lymphocytes/μl	2615 ± 900 (218) 2381 [2152–3078]	2624 ± 937 (187) 2430 [2237–3012]	ns	No
% $CD3^+CD4^+$ T lymphocytes	42.8 ± 4.5 (1.1) 43 [40.4–45.1]	38.5 ± 10.7 (2.6) 36.5 [32.9–44]	ns	No
% $CD3^+CD8^+$ T lymphocytes	39.3 ± 7.5 (1.8) 32.1 [25.4–33.2]	34.9 ± 10.3 (2.5) 33.9 [29.6–40.2]	ns	No
$CD4^+/CD8^+$ T cell ratio	1.4 ± 0.5 (0.1) 1.2 [1–1.7]	1 ± 0.6 (0.1) 0.9 [0.7–1.3]	ns	No
% $CD3^+CD4^+CD28^+$ lymphocytes	42.5 ± 6.3 (2) 41.1 [39.6–47.6]	34.5 ± 14.5 (2.9) 31.7 [24–44.3]	0.034	No
Number of $CD3^+CD4^+CD28^+$ lymphocytes/μl	1200 ± 511 (161) 1065 [834–1565]	960 ± 610 (125) 807 [702–1218]	ns	No
% $CD3^+CD20^+$ B lymphocytes	9.4 ± 5.1 (1.2) 7.6 [6.7–12]	9.1 ± 6.5 (1.4) 7.2 [6.1–12]	ns	No
% $CD3^-CD16^+CD56^+$ natural killer (NK) cells	11.8 ± 4.5 (1.1) 11 [7.6–17]	6.9 ± 5.8 (1.3) 5.7 [3.6–8.6]	0.001	Yes (weak)
Number of $CD3^-CD16^+CD56^+$ NK cells/μl	309 ± 149 (36) 298 [189–395]	185 ± 197 (40) 153 [87–233]	0.001	Yes (weak)
% $CD3^+CD16^+$ natural killer T (NKT) cells	7.7 ± 4.1 (1.0) 6.4 [4.5–10.7]	5.1 ± 5.5 (1.2) 3.3 [2–6.1]	0.009	No
Number of $CD3^-CD16^+$NKT cells/μl	191 ± 98 (24) 204 [110–240]	138 ± 179 (38) 76 [40–181]	0.017	No

ns = Not significant.
[a] See Section 2 for details.

Author's personal copy

Table 4
Distribution of peripheral regulatory T cell subsets in IPF patients.

Parameter	CTR (n = 17)	IPF (n = 29)	p[a]	Significance after multiple test correction
% $CD3^+CD4^+CD25^{high}FoxP3^+$ (Treg) cells	0.4 ± 0.2 (0.04) 0.4 [0.2–0.5]	0.68 ± 0.3 (0.06) 0.6 [0.5–0.8]	0.000	Yes (strong)
Number of Treg cells/μl	7.2 ± 4.1 (1.0) 5.8 [3.3–10.9]	18.0 ± 10.8 (2.1) 15.5 [10.7–23.9]	0.000	Yes (strong)
% $CD45RA^+$ T reg cells (naive)	7.0 ± 4.4 (1.0) 7.2 [3.9–9.8]	4.8 ± 3.3 (0.7) 4.3 [2.6–6.6]	ns	No
% $CD45RO^+$ Treg cells (effectors)	91.7 ± 5.9 (1.4) 92.4 [88.7–96.1]	91.9 ± 9.6 (1.9) 94 [91.2–97]	ns	No
% $CTLA\text{-}4^+$ Treg cells	74.1 ± 10.9 (3.0) 72.5 [48–77.6]	62.8 ± 29.7 (7.0) 72.8 [48–77.6]	ns	No
% $CD62L^+$ Treg cells	66.8 ± 15.9 (4.4) 63.3 [57.2–76.5]	66.1 ± 19.3 (4.4) 71.1 [56.8–75.4]	ns	No
% $CXCR4^+$ Treg cells	52.1 ± 27.3 (7.8) 63.4 [34.8–69.5]	36.3 ± 25.9 (5.4) 32.5 [25–47.5]	ns	No

ns = Not significant.
[a] See Section 2 for details.

4. Discussion

Aim of the present study was to investigate the distribution of peripheral immune cell subsets in patients affected by clinically stable idiopathic pulmonary fibrosis. Interestingly, we found that both the percentage and absolute number of NK cells were significantly reduced in IPF patients. This finding is in contrast with a previous report showing that peripheral NK cells were increased in a small cohort of 11 IPF patients [18]. Result differences may be explained, at least in part, by the adoption of different criteria for diagnosing IPF and by the inclusion as control group of patients affected by other interstitial lung diseases instead of healthy subjects. The sample size may also have been an issue of concern. In the present study, we further observed that similar perturbations, although at a lesser extent, were also applicable to NKT cells. Actually, there are no information addressing dynamics of NK and NKT cells in IPF pathogenesis for any comparison with our data; conversely, depletion of these cell subsets has been more extensively investigated in cancer biology [19,20]. Overall, analysis of $CD4^+$ and $CD8^+$ T cells and of B lymphocytes showed the lack of significant differences among IPF and control subjects.

To the best of our knowledge, this is also the first research addressing the distribution of circulating regulatory T lymphocytes, Th17 cells, and their balance in IPF patients. Despite these

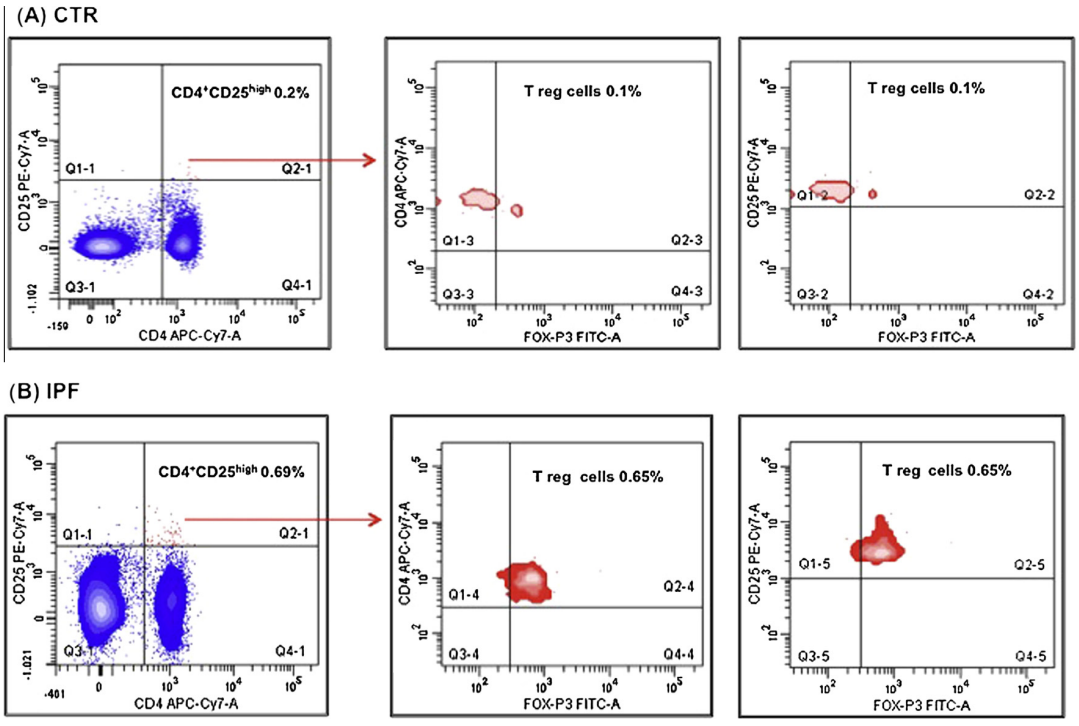
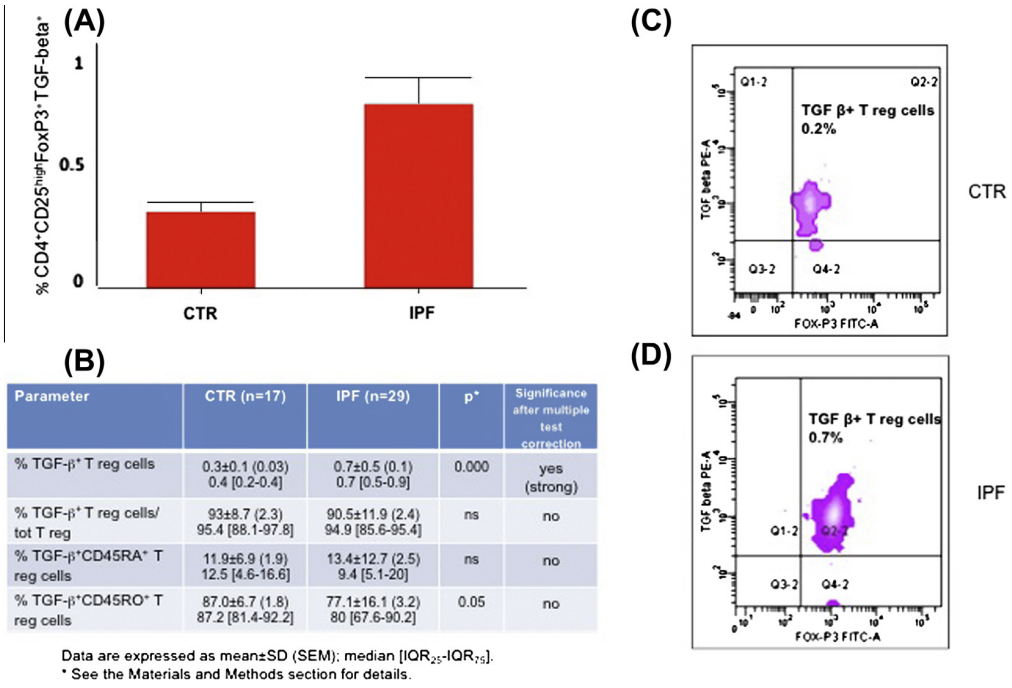


Fig. 1. Counter dot plots representing percentages of Treg cells in a control subject (A) and in IPF patient (B). Total lymphocytes (previously selected according to forward and side scatter parameters and CD3 expression) were gated on CD4 and CD25. $CD4^+CD25^{high}$ T cells were then gated on FOX-P3, as Treg cells. Frequencies of $CD4^+CD25^{high}$ FOX-P3 T cells are reported as percentage of total lymphocytes.

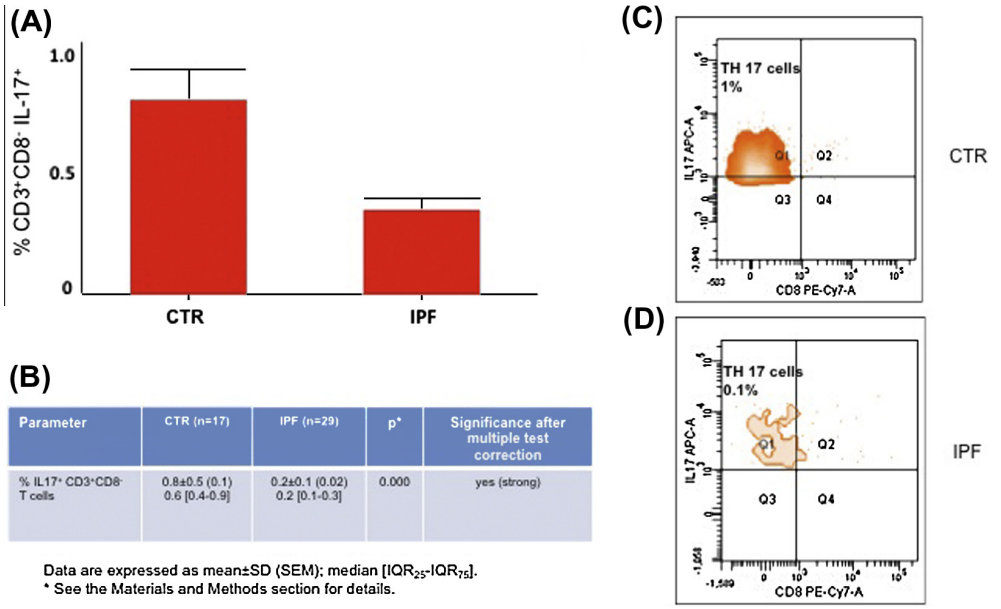
Author's personal copy



Parameter	CTR (n=17)	IPF (n=29)	p*	Significance after multiple test correction
% TGF-β+ T reg cells	0.3±0.1 (0.03) 0.4 [0.2-0.4]	0.7±0.5 (0.1) 0.7 [0.5-0.9]	0.000	yes (strong)
% TGF-β+ T reg cells/ tot T reg	93±8.7 (2.3) 95.4 [88.1-97.8]	90.5±11.9 (2.4) 94.9 [85.6-95.4]	ns	no
% TGF-β+CD45RA+ T reg cells	11.9±6.9 (1.9) 12.5 [4.6-16.6]	13.4±12.7 (2.5) 9.4 [5.1-20]	ns	no
% TGF-β+CD45RO+ T reg cells	87.0±6.7 (1.8) 87.2 [81.4-92.2]	77.1±16.1 (3.2) 80 [67.6-90.2]	0.05	no

Data are expressed as mean±SD (SEM); median [IQR_{25}-IQR_{75}].
* See the Materials and Methods section for details.

Fig. 2. Distribution and analytical data of peripheral TGF-beta expressing Treg cells are reported in IPF patients in comparison with control subjects in panels A and B, respectively. Representative flow cytometry counter plots in a healthy control (panel C) and in a IPF patient (panel D) are also shown. Numbers in the dot plots indicate the percentages of TGF-beta positive Treg cells.



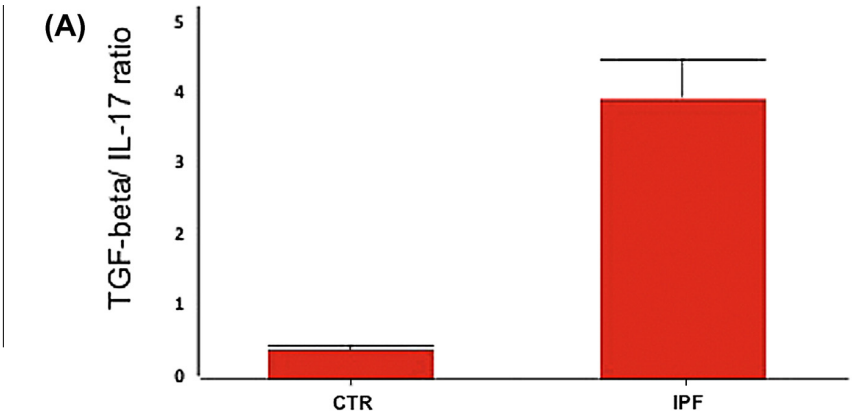
Parameter	CTR (n=17)	IPF (n=29)	p*	Significance after multiple test correction
% IL17+ CD3+CD8- T cells	0.8±0.5 (0.1) 0.6 [0.4-0.9]	0.2±0.1 (0.02) 0.2 [0.1-0.3]	0.000	yes (strong)

Data are expressed as mean±SD (SEM); median [IQR_{25}-IQR_{75}].
* See the Materials and Methods section for details.

Fig. 3. Distribution and analytical data of peripheral Th17 cells are reported in IPF patients in comparison with control subjects in panels A and B, respectively. Representative flow cytometry counter plots in a healthy control (panel C) and in a IPF patient (panel D) are also shown. Numbers in the dot plots indicate the percentages of IL-17 positive $CD3^+CD8^-$ T cells.

two novel T cell subpopulations share a common origin, they have a reciprocal relationship as exert mutually antagonistic functions. To date, the role of Treg cells in the pathogenesis of IPF is a matter of debate due to controversial results emerging from still limited evidence, at least in humans. Difficulties in this context are further increased due to the lack of an animal model strictly representative of human IPF. In this issue, recent data suggest regulatory T cells to exert differential roles in the animal model of bleomycin-induced fibrosis along with disease progression. Indeed, as reported by Boveda-Ruiz et al., Treg cells seem to be detrimental in early stages of lung disease, but protective later on [21]. In agreement with these findings, also Trujillo et al. have postulated Treg cells to be beneficial to the host in an attenuated form of bleomycin-related lung fibrosis induced in CCR7 deficient mice [22]. Similarly, Treg protec-

Author's personal copy



(B)

Parameter	CTR (n=17)	IPF (n=29)	p*	Significance after multiple test correction
TGF-β/IL-17 ratio	0.5±0.3 (0.08) 0.5 [0.3-0.7]	4.2±2.3 (0.46) 3.4 [2.2-6.4]	0.000	yes (strong)

Data are expressed as mean±SD (SEM); median [IQR_{25}-IQR_{75}].
* See the Materials and Methods section for details.

Fig. 4. Distribution and analytical data of the peripheral TGF-beta/IL17 ratio are reported in IPF patients in comparison with control subjects in panels A and B, respectively.

tion was already been reported in the *S.rectivirgula*-induced hypersensitivity pneumonitis animal model, by suppressing the production of interferon (IFN)-γ by $CD4^+$ and $CD8^+$ T cells [23].

We found that both the frequency and absolute number of blood $CD4^+CD25^{high}Foxp3^+$ cells were significantly increased in our cohort of clinically stable IPF cases when compared to normal subjects. This observation accounted for an increased proportion of cells expressing TGF-beta in IPF patients. Ability to express TGF-beta was however comparable to that of control subjects as almost the entire pool of Treg cells (>90%) was functionally recruited upon stimulation in both study groups. Distribution of Treg subsets, that are naïve ($CD45RA^+$) and effector/mature ($CD45RO^+$) cells, was quite similar in controls and IPF patients and equally contributed to the expression of TGF-beta. In a similar manner, we found no substantial differences when looking at the expression of surface markers of homing (CXCR4 and CD62L) and of suppressive function (CTLA-4), overall suggesting similar dynamics in cell turn-over and activation. In disagreement with our data, Kotsianidis et al. have recently shown that broncho-alveolar lavage (BAL) and peripheral blood (PB) $CD4^+CD25^+$ natural regulatory T cell numbers were reduced in IPF patients compared with those of healthy volunteers and patients without IPF (including patients with connective vascular disease-related interstitial pneumonitis) [13]. However, comparisons among study groups were not always statistically relevant. In addition, they reported that the suppressive potential of such cells was significantly compromised, BAL Treg cell activity being highly correlated with parameters of disease severity (with correlation analysis performed only in a small sample of IPF patients). Similarities in Treg cells impairment and IPF severity have been reported also by Shimizu et al. [14]. Further similar informations are available in different additional models of human lung fibrosis, not necessarily sharing common underlying pathways, which include granulomatous processes, like berillios and sarcoidosis [24–26], and autoimmune disorders with pulmonary involvement [27,28]. Apart from differences in technical procedures, a main limitation of our study that does not allow any comparison with literature reports is certainly represented by the lack of data collected at the BAL level. Overall, in our opinion, there is still not sufficient information for definitively allocate Treg cells in the unresolved puzzle of IPF pathogenesis.

By far, there is no information concerning the role and distribution of Th17 cells in this issue, whereas these cells have been more largely studied in autoimmune disorders with lung involvement/fibrosis, as discussed below. In healthy individuals, approximately 1% of PB $CD4^+$ T lymphocytes is represented by Th17 cells [29]. Interestingly, we found that the frequency of IL-17 expressing $CD4^+$ helper cells was dramatically reduced in IPF patients in comparison with the control group. Conversely, increased frequencies of Th17 cells along with Th22 lymphocytes (belonging to the Th17 cell family) have been reported in systemic sclerosis, the latter being associated as novel biomarker with interstitial lung disease [30]. Similarly, a skewed distribution of Th17 cells was suggested in a previous report to be involved in the pathogenesis of Wegener's granulomatosis, as they were found to be increased in patients with active disease but not in those with disease in remission [31]. Finally, in an animal model of silica-induced fibrosis, the contribution to disease progression of Th17 cells was pivotal for the induction of early inflammation, while it appeared to be dispensable for the development of the fibrotic response later on [32]. As a consequence of Th17 depletion, we further observed that IPF patients exhibited a functional imbalance of the Treg/Th17 axis that resulted to be increased by 8-times. In our opinion, this finding is of crucial importance since it is plausible to assume that, as for the Th1/Th2 dichotomy, any even subtle variation of the Treg/Th17 interplay may lead to significant perturbations of the immune response thus affecting the natural history of a given disease. This is also the case in patients affected by different types of cancer where the increased Treg/Th17 ratio has been recently shown to be negatively correlated with the disease stages [33–37]. Conversely, loss of balance between Th17 and regulatory T lymphocytes toward an increase of the former cells have been described, for instance, to occur more likely in inflammation-

Author's personal copy

related diseases like HIV infection [38], and in autoimmune disorders, like myasthenia gravis, Kawasaki disease, and rheumatoid arthritis [39–41].

5. Conclusions

Altogether, our data argue against the hypothesis that pathogenesis of IPF, no more considered as an inflammatory disorder, may be driven by an autoimmune process (as suggested by others). Depletion of NK and Th17 cells along with a not compromised Treg compartment delineate the existence of an "immune profile" in IPF that may be more closely assimilated to that observed in the cancer model. This is not surprising due to the poor prognosis of such a disease and to the widely recognized unresponsiveness to drugs with anti-inflammatory and/or immuno-suppressive activity. Further efforts in larger study populations are requested to address in a more precise fashion the contribution and dynamics of these critical players of the immune response in IPF. Their lung distribution and turn-over also need to be studied, as differences in cell compartmentalization cannot actually be rule out. This may help researchers and clinicians to identify predictive markers of disease phenotypes and outcomes and to improve the tailored development of future therapeutic strategies.

Authors contributions

D.G., A.T. and M.N. performed the experiments. M.D.M., G.C., A.A.S. and A.S. enrolled the patients. G.R. reviewed all radiological data. D.B. performed statistical analysis. D.G. and M.B. designed the study. M.B. enrolled the patients, coordinated the study and wrote the paper.

References

[1] Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2006;174:810–6.

[2] Ozawa Y, Suda T, Naito T, Enomoto N, Hashimoto D, Fujisawa T, et al. Cumulative incidence of and predictive factors for lung cancer in IPF. Respirology 2009;14:723–8. http://dx.doi.org/10.1111/j.1440-1843.2009.01547.x.

[3] Selman M, Pardo A. Idiopathic pulmonary fibrosis: an epithelial/fibroblastic cross-talk disorder. Respir Res 2002;3:3.

[4] King Jr TE, Schwarz MI, Brown K, Tooze JA, Colby TV, Waldron Jr JA, et al. Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. Am J Respir Crit Care Med 2001;164:1025–32.

[5] Bocchino M, Agnese S, Fagone E, Svegliati S, Grieco D, Vancheri C, et al. Reactive oxygen species are required for maintenance and differentiation of primary lung fibroblasts in idiopathic pulmonary fibrosis. PLoS One 2010;5:e14003.

[6] Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3$^+$ regulatory T cells in the human immune system. Nat Rev Immunol 2010;10:490–500.

[7] Baratelli F, Lee JM, Hazra S, Lin Y, Walser TC, Schaue D, et al. PGE2 contributes to TGF-β induced T regulatory cell function in human non-small cell lung cancer. Am J Transl Res 2010;2:356–67.

[8] Wilke CM, Bishop K, Fox D, Zou W. Deciphering the role of Th17 cells in human disease. Trends Immunol 2011;32:603–11.

[9] Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, et al. Th17 cells promote cytotoxic T cell activation in tumor immunity. Immunity 2009;31:787–98.

[10] Kryczek I, Wei S, Szeliga W, Vatan L, Zou W. Endogenous IL-17 contributes to reduced tumor growth and metastasis. Blood 2009;114:357–9.

[11] Vancheri C, Failla M, Crimi N, Raghu G. Idiopathic pulmonary fibrosis: a disease with similarities and links to cancer biology. Eur Respir J 2010;35:496–504.

[12] Feghali-Bostwick CA, Tsai CG, Valentine VG, Kantrow S, Stoner MW, Pilewski JM, et al. Cellular and humoral autoreactivity in idiopathic pulmonary fibrosis. J Immunol 2007;179:2592–9.

[13] Kotsianidis I, Nakou E, Bouchliou I, Tzouvelekis A, Spanoudakis E, Steiropoulos P, et al. CD4$^+$CD25$^+$FOXP3$^+$ regulatory T cells in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2009;179:1121–30.

[14] Shimizu Y, Dobashi K, Endou K, Ono A, Yanagitani N, Utsugi M, et al. Decreased interstitial FOXP3(+) lymphocytes in usual interstitial pneumonia with discrepancy of CXCL12/CXCR4 axis. Int J Immunopathol Pharmacol 2010;23:449–61.

[15] Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med 2011;183:788–824.

[16] Guo W. A note on adaptive Bonferroni and Holm procedures under dependence. Biometrika 2009;96:1012–8.

[17] Gilani SR, Vuga LJ, Lindell KO, Gibson KF, Xue J, Kaminski N, et al. CD28 down-regulation on circulating CD4 T cells is associated with poor prognosis of patients with idiopathic pulmonary fibrosis. PLoS One 2010;5:e8959.

[18] Esposito I, Perna F, Ponticiello A, Perrella M, Gilli M, Sanduzzi A. Natural killer cells in Bal and peripheral blood of patients with idiopathic pulmonary fibrosis (IPF). Int J Immunopathol 2005;18:541–5.

[19] Salagianni M, Baxevanis CN, Papamichail M, Perez SA. New insights into the role of NK cells in cancer immunotherapy. Oncoimmunology 2012;1:205–7.

[20] Pilones KA, Aryankalayil J, Demaria S. Invariant NKT cells as novel targets for immunotherapy in solid tumors. Clin Dev Immunol 2012;2012:720803. http://dx.doi.org/10.1155/2012/720803 [Epub 2012 October 17].

[21] Boveda-Ruiz D, D'Alessandro-Gabazza CN, Toda M, Takagi T, Naito M, Matsushima Y, et al. Differential role of regulatory T cells in early and late stages of pulmonary fibrosis. Immunobiology 2013;218:245–54.

[22] Trujillo G, Hartigan AJ, Hogaboam CM. T regulatory cells and attenuated bleomycin-induced fibrosis in lungs of CCR7−/− mice. Fibrogenesis Tissue Repair 2010;3:18. http://dx.doi.org/10.1186/1755-1536-3-1.

[23] Park Y, Oh SJ, Chung DH. CD4$^+$CD25$^+$ regulatory T cells attenuate hypersensitivity pneumonitis by suppressing IFN-γ production by CD4$^+$ and CD8$^+$ T cells. J Leuk Biol 2009;86:1427–37.

[24] Mack DG, Lanham AM, Falta MT, Palmer BE, Maier LA. Deficient and dysfunctional regulatory T cells in the lungs of chronic beryllium disease subjects. Am J Respir Crit Care Med 2010;181:1241–9.

[25] Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. The immune paradox of sarcoidosis and regulatory T cells. J Exp Med 2006;203:359–70.

[26] Idali F, Wahlström J, Müller-Suur C, Eklund A, Grunewald J. Analysis of regulatory T cells associated forkhead fox P3 expression in the lungs of patients with sarcoidosis. Clin Exp Immunol 2008;152:127–37.

[27] Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 2005;175:8392–400.

[28] Lee HY, Hong YK, Yun HJ, Kim YM, Kim JR, Yoo WH. Altered frequency and migration capacity of CD4$^+$CD25$^+$ regulatory T cells in systemic lupus erythematosus. Rheumathology 2008;47:789–94.

[29] Wilkie CA, Bishop K, Fox D, Zou W. Deciphering the role of Th17 cells in human disease. Cell 2011;32:603–11.

[30] Truchetet ME, Brembilla NC, Montanari E, Allanore Y, Chizzolini C. Increased frequency of circulating Th22 in addition to Th17 and Th2 lymphocytes in systemic sclerosis: association with interstitial lung disease. Arthritis Res Ther 2011;13:R166.

[31] Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CGM. Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission. Arthritis Rheum 2008;58:2196–205.

[32] Lo Re S, Dumoutier L, Couillin I, Van Vyve C, Yakoub Y, Uwambayinema F, et al. IL-17A-producing γδT and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. J Immunol 2010;184:6367–77.

[33] Sfanos KS, Bruno TC, Maris CH, Xu L, Thoburn CJ, De Marzo AM, et al. Phenotypic analysis of prostate-infiltrating lymphocytes reveals TH17 and Treg skewing. Clin Cancer Res 2008;14:3254–61.

[34] Chi LJ, Lu HT, Li GL, Wang XM, Su Y, Xu WH, et al. Involvement of T helper type 17 and regulatory T cell activity in tumour immunology of bladder carcinoma. Clin Exp Immunol 2010;161:480–9.

[35] Maruyama T, Kono K, Mizukami Y, Kawaguchi Y, Mimura K, Watanabe M, et al. Distribution of Th17 cells and FoxP3(+) regulatory T cells in tumor-infiltrating lymphocytes, tumor-draining lymph nodes and peripheral blood lymphocytes in patients with gastric cancer. Cancer Sci 2010;101:1947–54.

[36] Wang J, Cai D, Ma B, Wu G, Wu J. Skewing the balance of regulatory T-cells and T-helper 17 cells in breast cancer patients. J Int Med Res 2011;39:691–701.

[37] Zhao L, Yan J, Wang HP, Liu RY. Imbalance in the Th17/Treg and cytokine environment in peripheral blood of patients with adenocarcinoma and squamous cell carcinoma. Med Oncol 2013;30:461. http://dx.doi.org/10.1007/s12032-013-0461-7 [Epub 2013 January 20].

[38] Di L, Chen J, Jia M, Hong K, Ruan Y, Liang H, et al. Loss of balance between T helper type 17 and regulatory T cells in chronic human immunodeficiency virus infection. Clin Exp Immunol 2011;165:363–71.

[39] Masuda M, Matsumoto M, Tanaka S, Nakajima K, Yamada N, Ido N, et al. Clinical implication of peripheral CD4$^+$CD25$^+$ regulatory T cells and Th17 cells in myasthenia gravis patients. J Neuroimmunol 2010;225:123–31.

[40] Jia S, Li C, Wang G, Yang J, Zu Y. The T helper type 17/regulatory T cell imbalance in patients with acute Kawasaki disease. Clin Exp Immunol 2010;162:131–7.

[41] Wang W, Shao S, Jiao Z, Guo M, Xu H, Wang S. The Th17/Treg imbalance and cytokine environmental in peripheral blood of patients with rheumatoid arthritis. Rheumatol Int 2012;32:887–93. http://dx.doi.org/10.1007/s00296-010-1710-0 [Epub 2011 January 11].