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CCR5 expression and CC chemokine levels in idiopathic pulmonary fibrosis

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ABSTRACT: CC chemokines play an important role in the pathogenetic mechanisms of interstitial lung disease, while a downregulation of CC chemokine receptor (CCR)5 in the fibrotic stages of sarcoidosis has been observed.

To evaluate the involvement of CC chemokines and the expression of CCR5 in idiopathic pulmonary fibrosis (IPF) and, more specifically, in usual interstitial pneumonia, 35 subjects were studied. CC chemokine ligand (CCL)2, CCL3 and CCL4 levels were measured in the bronchoalveolar lavage fluid (BALF) of 18 nonsmoker control subjects and 17 patients affected by IPF. CCR5 expression was evaluated in alveolar macrophages and lymphocytes.

The BALF levels of all chemokines were significantly increased in IPF: median (range) CCL3 1.6 (1.0–11.1) *versus* 1.2 (0.0–3.8) pg·mL^{-1}; CCL4 6.2 (1.3–96.0) *versus* 3.4 (0.3–6.8) pg·mL^{-1}; and CCL2 60.1 (16.7–251.3) *versus* 4.6 (0.5–119.4) pg·mL^{-1}. CCL2 levels correlated negatively with the carbon monoxide diffusing capacity of the lung ($D_{L,CO}$) and arterial oxygen tension. CCR5 expression was significantly reduced in lymphocytes from IPF compared with controls.

The CC chemokines investigated are involved in the inflammatory mechanisms of idiopathic pulmonary fibrosis, and the results are in agreement with the hypothesis of a downregulation of the T-helper 1 immunological response in this disease.

KEYWORDS: Bronchoalveolar lavage, CC chemokine, CC chemokine receptor 5, idiopathic pulmonary fibrosis

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Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic fibrosing interstitial pneumonia limited to the lung, with the histopathological characteristics of usual interstitial pneumonia (UIP) on lung biopsy [1]. The aetiology is unknown. The histological hallmark and chief diagnostic criterion is a heterogeneous appearance at low magnification, with alternating areas of normal lung, interstitial inflammation, fibrosis and honeycomb change [1].

Normal repair following lung injury results in the rapid restoration of tissue integrity and function. In contrast to normal repair, chronic inflammation in IPF promotes fibroproliferation and deposition of extracellular matrix, reflecting disregulated and exaggerated tissue repair. A salient feature of chronic inflammation is infiltration by leukocytes, and their recruitment requires intercellular communication between infiltrating leukocytes on the one hand, and the endothelium and resident stromal and parenchymal cells on the other. These events are mediated by the generation of early-response cytokines, such as interleukin (IL)-1 and tumour necrosis factor (TNF), the expression of cell-surface adhesion molecules, and the production of chemotactic molecules, such as chemokines [2].

Originally described as chemotactic factors, chemokines are now known to modulate cytokine production, adhesion molecule expression and mononuclear cell proliferation [3]. In animal models of IPF induced by bleomycin, CC chemokines such as CC chemokine ligand (CCL)3 (macrophage inflammatory protein (MIP)-1α) and CCL2 (monocyte chemotactic protein-1) have been shown to play a critical role in the initiation and maintenance of pulmonary lesions and their angiogenesis [3].

No data are available on the role of CCL4 (MIP-1β) in IPF, but an augmented production of CCL4 by peripheral blood mononuclear cells and increased serum concentrations of this chemokine, together with CCL2 and CCL3, have been observed in systemic sclerosis involving the lung [4].

The balance between two macrophage-derived CC chemokines, CCL3 and CCL4, could potentially be involved in sustaining inflammation and a chronic course in IPF. Both of these chemokines

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recognise CC chemokine receptor (CCR)5 as a cellular receptor in activated T-lymphocytes and alveolar macrophages [5]. Stimulation of CCR5 induces an intracellular biochemical cascade, with increased production and release of IL-2 and interferon (IFN)-γ [6].

In a recent study on pulmonary sarcoidosis, an increase of CCR5 expression was observed in lymphocytes and alveolar macrophages of patients affected by sarcoidosis, but with a downregulation in the advanced stage; an involvement of CCL4 from the earliest phases of the disease was also observed, with an involvement of CCL3 prevalently in the advanced fibrotic stages of sarcoidosis [7]. To compare IPF with the advanced stage of sarcoidosis in the present study, the authors measured the levels of CCL3 and CCL4 in bronchoalveolar lavage (BAL) of patients affected by IPF and the expression of their receptor CCR5 in alveolar cells. The BAL fluid (BALF) concentrations of CCL2 and their correlations with the degree of functional impairment were also evaluated. Some results of this study have been previously reported in the form of an abstract [8].

METHODS

Study population

Eighteen healthy, nonsmoker historical controls, who presented also in a previous study by the current authors [7], and 17 patients affected by mild–moderate IPF were studied. IPF was diagnosed on the basis of clinical, functional, radiographic and histological criteria. According to the definition of IPF by the American Thoracic Society and European Respiratory Society international consensus statement, nine patients had histological evidence of UIP at biopsy obtained with thoracotomy or video-assisted thoracoscopy, and eight patients were diagnosed as IPF with transbronchial biopsy and BAL in the presence of all major and minor diagnostic criteria [1]. Nine patients were nonsmokers and eight were ex-smokers (for $\geqslant$5 yrs). Treatment with steroid or nonsteroid anti-inflammatory drugs was stopped $\geqslant$3 months before inclusion in the study. None of the subjects had ever been prescribed cytotoxic or immunomodulatory drug therapy before inclusion.

Informed consent was obtained from each subject, and the protocol was approved by the Review Board of the Scientific Institute of Veruno, Veruno, Italy.

Pulmonary function tests

Pulmonary function testing included the measurement of forced expiratory volume in one second, forced vital capacity (6200 Autobox Pulmonary Function Laboratory; Sensormedics Corp., Yorba Linda, CA, USA) and carbon monoxide diffusing capacity of the lung ($D_{L,CO}$), determined by the single-breath method (2200 Pulmonary Function Laboratory; Sensormedics Corp.).

Processing of bronchoalveolar lavages

BAL was performed as previously described [9]. Briefly, three 50-mL boluses of 37℃ pre-warmed sterile 0.9% saline were instilled in the right middle lobe and recovered by syringe with normal suction. Fluids were filtered through a monolayer of sterile gauze. Cytocentrifuge (Cytospin II; Shandon, London, UK) slides, prepared with native fluid, were stained with May-Grunwald Giemsa to determine the cell differentials. A total of 500 cells (without epithelial cells) per slide was examined at $\times$1,000 magnification. The cytocentrifuge slides used for immunocytochemistry were fixed for 2 min with acetone and frozen at -80℃ until analysis. BAL supernatants obtained after refrigerated (4℃) centrifugation were immediately stored at -80℃ until analysis.

Immunocytochemistry

The expression of CCR5 in BAL cells was assessed by immunocytochemistry on frozen cytocentrifuge slides. The anti-CCR5 (MAB 181) was purchased from R&D Systems (Abington, UK). Monoclonal antibody binding was detected with the alkaline phosphatase anti-alkaline phosphatase method (APAAP kit system K670; Dako, Copenhagen, Denmark) and fast-red substrate.

Cytospin preparations of lipopolysaccharide-stimulated mononuclear cells were used as a positive control in each staining run. Negative control slides included cytospin preparations immunostained with mouse monoclonal immunoglobulin G2a (Dako).

Light-microscopic analysis of stained cells was performed at a magnification of $\times$1,000 and a total of $\geqslant$250 cells (macrophages and lymphocytes) was examined and identified on the basis of morphological criteria.

Immunoassay

The levels of the chemokines in BALF, concentrated at least 10-fold with Centricon-3 concentrators (Amicon Inc., Beverly, MA, USA), were obtained with Quantikine CCL2, CCL3 and CCL4 ELISA kits (R&D Systems). This assay employs the quantitative sandwich-enzyme immunoassay technique. A monoclonal antibody specific for the chemokine is pre-coated onto a microtitre plate. The standard curves were prepared from 0 to 1,000 pg·mL^{-1}. The lower limits of detection of the kits (manufacturer's data) were 5.0, 6.0 and 4.0 pg·mL^{-1} for CCL2, CCL3 and CCL4, respectively. Standards and samples were run in duplicate. The results obtained were divided for the concentration value.

Statistical analysis

Demographic and functional data are reported as mean$\pm$SD; the other results are expressed as median (range). Differences between the two groups were assessed with an unpaired t-test for demographic and functional data, and Mann-Whitney U-tests for the other results. Spearman's rank correlation coefficient was used to study correlations between chemokine concentrations and both pulmonary function tests and BAL cellular populations. Statistical significance was defined as $p<0.05$.

RESULTS

Functional data

Table 1 shows the demographic and functional data of the study population. IPF patients were significantly older than control subjects. Restriction in pulmonary function tests and impaired gas exchange were observed in all IPF patients with a $D_{L,CO}$ of 52.2$\pm$13.9% and an arterial oxygen tension (P_{a,O_2}) of 9.0$\pm$1.4 kPa, compared with 98.3$\pm$10.3% and 11.9$\pm$0.3 kPa in controls, respectively.

TABLE 1 Demographic and functional data of the study population

	Controls	IPF	p-values#
Subjects n	18	17	
Males/females	11/7	7/10	
Age	46.6±20.5	62.7±10.1	0.0005
FEV_1 % pred	106.4±16.7	77.4±18.1	0.0001
FVC % pred	105.7±14.8	74.1±18.5	<0.0001
FEV_1/FVC	85.7±6.5	93.9±7.2	0.01
$D_{L,CO}$ %	98.3±10.3	52.2±13.9	<0.0001
P_{a,O_2} mmHg	89.4±2.2	67.3±10.7	<0.0001

Data are presented as mean±SD, unless otherwise stated. IPF: idiopathic pulmonary fibrosis; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; $D_{L,CO}$: carbon monoxide diffusing capacity of the lung; P_{a,O_2}: arterial oxygen tension. #: using t-test. kPa=0.133×mmHg.

Differential cell counts of BALF

The BAL number of total cells was slightly but significantly increased in IPF (200.3×10^3 cells·mL^{-1} *versus* 125.1×10^3 cells·mL^{-1}; p<0.05) compared with controls, as shown in table 2. This increase consisted of both a per cent (p<0.0001) and absolute numerical (p<0.0001) increase of neutrophils and eosinophils. The median (range) number of macrophages in the BAL of control subjects and IPF patients was very similar (105.6 (46.4–234.1)×10^3 cells·mL^{-1} *versus* 119.0 (29.4–226.1)×10^3 cells·mL^{-1}; nonsignificant).

BALF levels of CCL2, CCL3 and CCL4

All the chemokines evaluated were increased in the BALF of IPF patients. The increase of CCL3 (fig. 1) was slightly, but statistically, significant compared with controls (1.73 (1.05–11.07) *versus* 1.22 (0.0–3.84) pg·mL^{-1}; p<0.03). More evident (fig. 2) was the difference between the two groups concerning the concentrations of CCL4 (7.03 (1.26–96.04) *versus* 3.10 (0.28–6.8) pg·mL^{-1}; p<0.003). As expected, the levels of CCL2 in the BALF of patients affected by IPF were clearly increased, as shown in figure 3, compared with controls (68.65 (16.7–251.3) *versus* 4.56 (0.50–119.4) pg·mL^{-1}; p<0.0001).

TABLE 2 Characteristics of bronchoalveolar lavage of the study population

	Controls	IPF	p-value#
Subjects n	18	17	
Recovery mL	84 (63–102)	77 (30–97)	0.66
Cells·mL^{-1} ×10^3	125.1 (54.6–245.4)	200.3 (68.4–271.8)	0.048
Macrophages %	90.5 (74.3–95.6)	81.6 (40.5–88.2)	0.0004
Lymphocytes %	8.5 (2.2–23.1)	4.8 (2.3–7.4)	0.27
Neutrophils %	1.5 (0.2–2.8)	10.0 (0.3–35.1)	<0.0001
Eosinophils %	0.3 (0–1.1)	3.5 (0.1–24.4)	<0.0001
Basophils %	0.1 (0–0.9)	0.1 (0–0.4)	0.59
Macrophages·mL^{-1} ×10^3	105.6 (46.4–234.1)	119.0 (29.4–226.1)	0.38
Lymphocytes·mL^{-1} ×10^3	8.0 (2.2–37.1)	7.4 (2.5–15.3)	0.90
Neutrophils·mL^{-1} ×10^3	1.7 (0.2–5.4)	17.9 (0.2–85.7)	<0.0001
Eosinophils·mL^{-1} ×10^3	0.5 (0–1.4)	4.3 (0.1–65.6)	<0.0001
Basophils·mL^{-1} ×10^3	0.1 (0–1.5)	0.1 (0–0.4)	0.57

Data are presented as median (range), unless otherwise stated. IPF: idiopathic pulmonary fibrosis. #: using Mann-Whitney U-test.

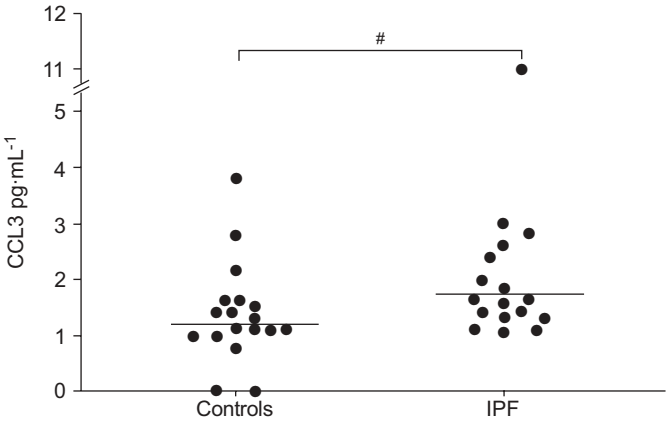

FIGURE 1. Concentrations of CC chemokine ligand (CCL)3 in the bronchoalveolar lavage fluid of controls and idiopathic pulmonary fibrosis (IPF) patients. Horizontal bars represent median values. #: p<0.03.

Relationship between functional impairment, BAL characteristics and concentrations of CCL2, CCL3 and CCL4

Since the two groups were significantly different, the current authors analysed the relationship between concentrations of the chemokines and both functional and cellular data within the IPF group alone. With control subjects excluded from the analysis, many statistically significant correlations disappeared. Nevertheless, the increase of CCL2 concentrations in the BALF of patients affected by IPF was correlated with P_{a,O_2} (p<0.003) and $D_{L,CO}$ (p<0.009), as shown in figure 4. No significant correlations were found comparing the concentrations of CCL4 and functional or cellular data. Conversely, as shown in figure 5, the levels of CCL3 in the BALF of IPF

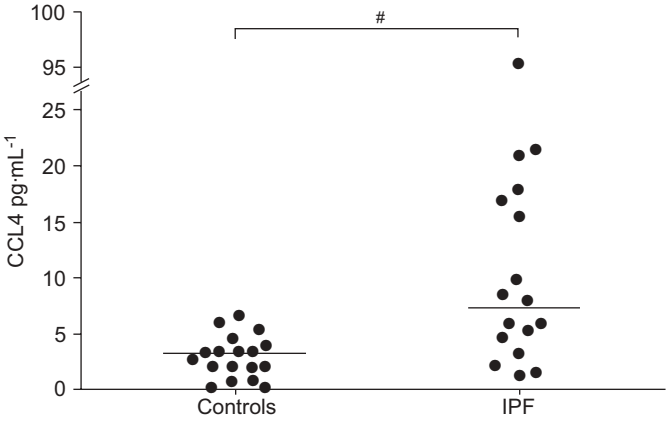

FIGURE 2. Concentrations of CC chemokine ligand (CCL)4 in the bronchoalveolar lavage fluid of controls and idiopathic pulmonary fibrosis (IPF) patients. Horizontal bars represent median values. #: p<0.003.

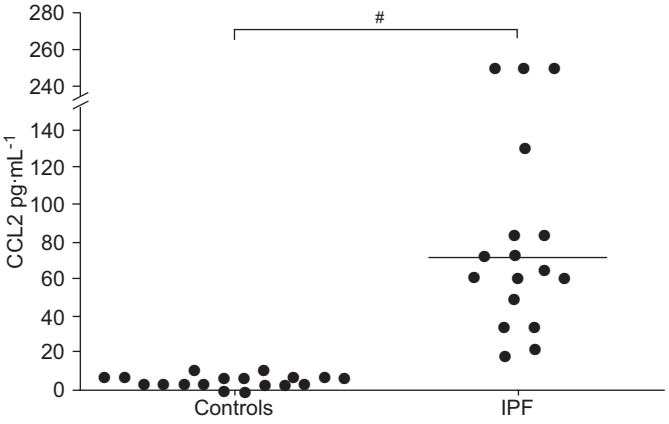

FIGURE 3. Concentrations of CC chemokine ligand (CCL)2 in the bronchoalveolar lavage fluid of controls and idiopathic pulmonary fibrosis (IPF) patients. Horizontal bars represent median values. #: $p<0.0001$.

patients correlated significantly with both percentage and total number per mL of neutrophils ($\rho=0.569$, $p<0.05$, and $\rho=0.608$, $p<0.02$, respectively) and eosinophils ($\rho=0.600$, $p<0.03$, and $\rho=0.675$, $p<0.02$, respectively).

CCR5-positive cells in BALF

The alveolar macrophages of IPF patients and controls expressed the chemokine receptor CCR5 in a similar way, as shown in table 3. Conversely, the CCR5+ lymphocytes were significantly lower in IPF patients than controls, both in percentage and absolute number ($p<0.002$ and $p<0.007$, respectively).

DISCUSSION

The current findings demonstrate increased levels of three CC chemokines, CCL2, CCL3 and CCL4, in the BALF of patients affected by IPF, associated with a decreased expression of cellular receptor CCR5 in lymphocytes.

The pathogenesis of IPF or UIP is due to inflammation/injury to the alveolar-capillary wall basement membrane, leading to a loss of type-I epithelial and endothelial cells, proliferation of type-II cells, a loss of alveolar integrity, recruitment and proliferation of stromal cells, and deposition of extracellular matrix and end-stage fibrosis [10]. McKee *et al*. [11] observed that hyaluronan fragments, glycosaminoglycan constituents of the extracellular matrix, are capable of activating macrophages and inducing the expression of genes whose functions are relevant to chronic inflammation, in particular, the chemokine gene family: CCL2, CCL3 and CCL4, cytokine responsive gene-2 and RANTES (regulated on activation, normal T-cell expressed and secreted) [11].

The involvement of CC chemokines in the inflammatory events of IPF has been observed in the increased levels of CCL2 and CCL3 in IPF, in the animal fluorescein isothiocyanate (FITC) and bleomycin models [3, 12] and in human studies [13]. Using both FITC and bleomycin models in mice, Moore *et al*. [12] observed that animals with genetically modified CCR2-/- were protected from the development of pulmonary fibrosis compared with the wild-type CCR2+/+, even if, in both types of mice, CCL2 levels in BAL were increased.

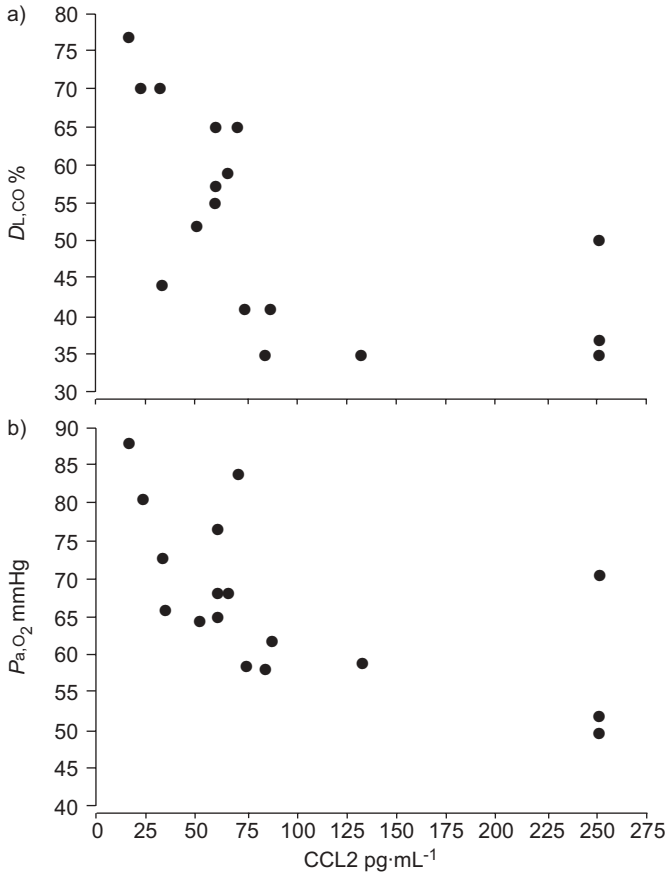

FIGURE 4. Relationship of CC chemokine ligand (CCL)2 concentrations in the bronchoalveolar lavage fluid of idiopathic pulmonary fibrosis patients with a) carbon monoxide diffusing capacity of the lung ($D_{L,CO}$; $\rho=-0.768$; $p=0.0021$) and b) arterial oxygen tension (P_{a,O_2}; $\rho=-0.654$; $p=0.009$). kPa = 0.133 × mmHg.

In humans, Suga *et al*. [13] observed increased levels of CCL2 in the BAL and serum of patients affected by IPF, and demonstrated a relationship between serum levels of CCL2 and the clinical course of interstitial lung disease. The current findings confirm the increased levels of CCL2 in the BAL of patients affected by IPF compared with normal subjects, and reinforce the relationship observed by Suga *et al*. [13] between this chemokine and the clinical course of the disease, showing a significant correlation also with the degree of functional impairment and, thus, with the severity of the disease.

CCL2 and its receptor CCR2 are involved in fibrosis through the regulation of profibrotic fibroblast-derived cytokine generation and matrix deposition: fibroblasts isolated and cultured from fibrotic granuloma (T-helper (Th)2-type lesion) generated twice as many CCL2, as did similar numbers of fibroblasts from nonfibrotic granuloma (Th1-type lesion) or normal fibroblasts. In addition, stimulation of IL-4, a typical Th2 cytokine, also increases the number of normal fibroblasts expressing CCR2; in contrast, treatment with IFN-γ, a Th1 cytokine, significantly decreases the number of CCR2-expressing normal and Th2-type fibroblasts [14]. In addition, IFN-γ can inhibit both fibroblast and chondrocyte collagen production *in vitro*, as well as decrease the expression of steady-state type-I and type-III procollagen mRNA; IFN-γ is

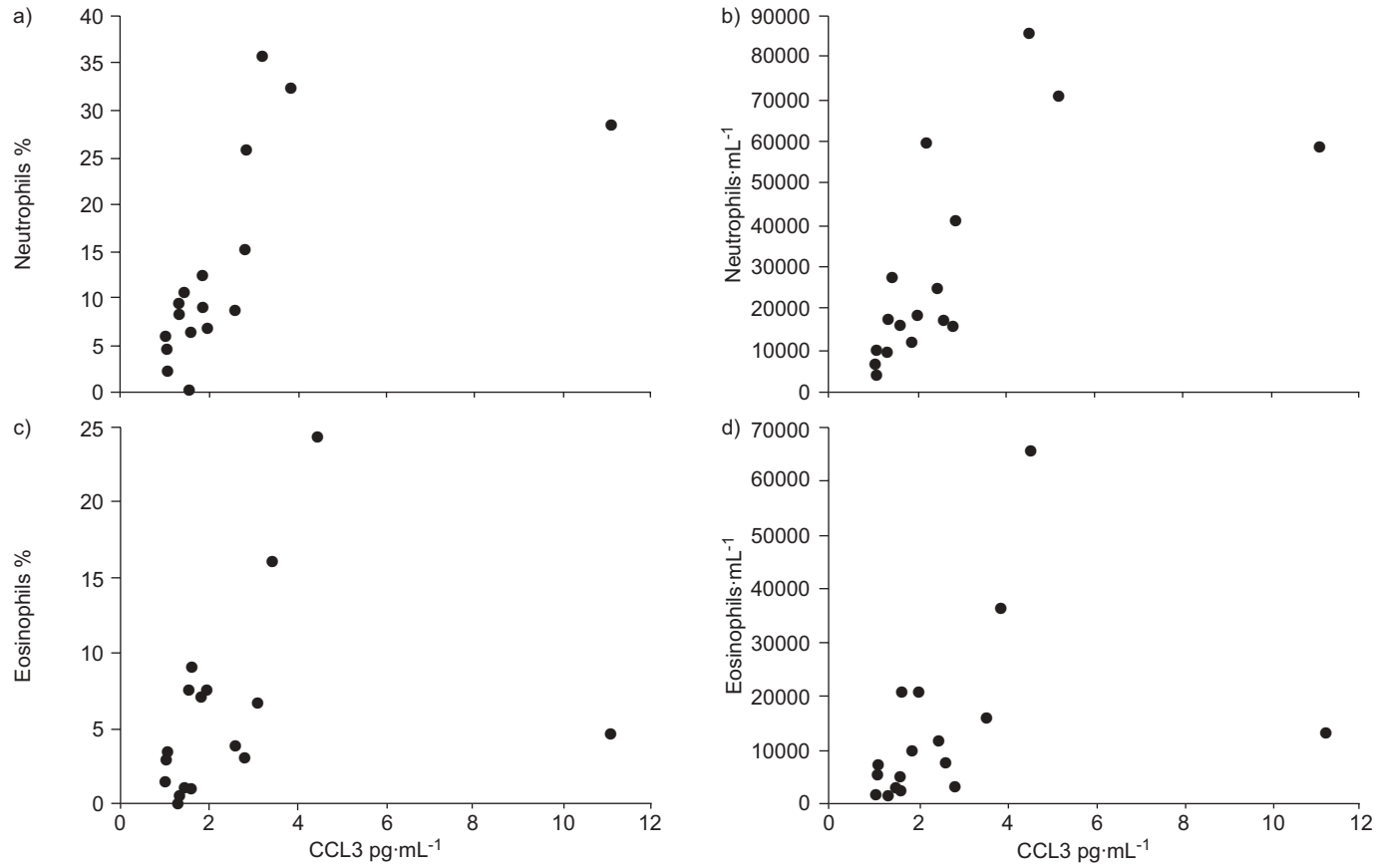

FIGURE 5. Relationship between CC chemokine ligand (CCL)3 concentrations in the bronchoalveolar lavage fluid of idiopathic pulmonary fibrosis and percentage (a, c) and number (b, d) of neutrophils and eosinophils (a: ρ=0.569, p=0.04; b: ρ=0.608, p=0.015; c: ρ=0.600, p=0.028; d: ρ=0.675, p=0.015).

one of the major type-I cytokines that possesses profound regulatory activity for collagen deposition during chronic inflammation [15, 16].

In addition, since the activation of CCR5 induces an increased production of IL-2 and IFN-γ [6], the downregulation of this receptor, which the current authors, for the first time, demonstrated in lymphocytes of IPF patients, could be correlated with a decrease in the levels of IFN-γ.

TABLE 3 T-cell subsets and CC chemokine receptor (CCR)5+ lymphocytes (Ly) and macrophages (Am) in bronchoalveolar lavage of the study population

	Controls	IPF	p-value
Subjects n	18	17	
CCR5+ Ly %	20.5 (2.0–40.0)	5.0 (0.0–30.0)	0.0015
CCR5+ Am %	2.8 (0.0–25.8)	2.3 (0.0–11.0)	0.61
CCR5+ Ly·mL^{-1} ×10^3	1.4 (0.1–5.3)	0.4 (0.0–2.0)	0.0062
CCR5+ Am·mL^{-1} ×10^3	2.4 (0.0–35.9)	5.1 (0.0–30.9)	0.38

Data are presented as median (range), unless otherwise stated. IPF: idiopathic pulmonary fibrosis.

Recently, whilst evaluating surgical biopsies, and isolating and culturing primary pulmonary fibroblast lines from UIP, CHOI *et al*. [17] observed an increased expression of CCR5 in these latter cells, but did not evaluate alveolar macrophages and lymphocytes. Interestingly, in the same study, CHOI *et al*. [17] also observed an increase of CCL7 levels in UIP, and reported that this chemokine could bind CCR5 with high affinity without eliciting a functional response and displacing CCR5 agonists. Hence, the presence of high levels of CCL7 could contribute to the reduced activity of CCR5 and, consequently, to the decreased production of IFN-γ. In support of these data, in recent studies on sarcoidosis [7, 18], the current authors observed a downregulation of CCR5 expression in lymphocytes and macrophages between the first and third (fibrotic) stages, coupled with an increase of IL-4 concentrations and decreased levels of IFN-γ in the BAL of patients with advanced disease compared with first and second stage. These data confirm the hypothesis that a persistent imbalance in the expression of Th2 *versus* Th1 cytokines in the lung acts as a mechanism for the progression of pulmonary fibrosis and that, in the advanced stages of sarcoidosis and pulmonary fibrosis, the type of inflammation is very similar, despite the fact that the initial disease is different. Less is known about the role of CCL3 and CCL4 in the inflammatory mechanism of IPF in humans.

An important role of CCL3 has been observed in the inflammatory process of fibrotic lung diseases and in the bleomycin-induced model of pulmonary fibrosis in mice [3, 19], in which treatment with anti-CCL3 antibody reduced the net lung hydroxyproline content by 49% compared with bleomycin-challenged mice treated with nonimmune serum [20]. In a study on sarcoidosis, the current authors observed a different involvement of these CC chemokines during the evolution towards a fibrotic advanced stage [7]. When evaluating groups of patients at different stages of sarcoidosis, an involvement of both these CC chemokines was observed: CCL4 from the earliest phases of the disease and CCL3 prevalently in the advanced fibrotic stages of sarcoidosis. In addition, a significant correlation was found between the levels of CCL3 in BAL and neutrophils [7].

In the present study, the authors confirmed increased levels of these two chemokines in the BAL of fibrotic patients and, as expected, an increase of the number and percentage of neutrophils, which positively correlate with CCL3 levels in BAL.

BLESS *et al*. [21] observed in rats that CCL4 contributed significantly to the recruitment of neutrophils, and to lung production of TNF-α, which is secreted by hyperplastic type-II alveolar cells in pulmonary fibrosis and promotes DNA synthesis and proliferation of fibroblasts [22].

No correlation was found between CCL4 levels and the number or percentage of neutrophils. In fact, in humans, this chemokine is not involved in neutrophil recruitment [23].

Finally, viral infection, in particular, herpes virus chronic infection, is one of the hypothetic aetiologies of UIP [15, 22]. LILLARD *et al*. [24] observed recently that both CCL3 and CCL4 are not only involved in inflammation, but they also mediate mucosal and systemic adaptive immunity, enhancing the mucosal and serum humoral as well as cellular immune response against infectious diseases, particularly those of a viral origin. CCR5 is a coreceptor for entry of HIV-1 infection in cells, CCL7 is increased in UIP and prevents the binding of R5 strains of HIV [17], while CCL3 and CCL4 are potent inhibitors of M-tropic HIV infection [24], and both, as observed in the present study, are increased in UIP. All these observations could contribute to reinforce the hypothesis of viral involvement in the aetiology of UIP.

Therefore, the role and function of chemokines in the inflammation and immunity of pulmonary disease, and, in particular, in the pathogenesis of interstitial lung diseases, is far from being completely clarified. The present data, however, suggest a profibrotic role of CC chemokines in the progression of idiopathic pulmonary fibrosis and contribute, as understanding of the inflammatory mechanism underlying usual interstitial pneumonia/idiopathic pulmonary fibrosis increases, towards the development of possible approaches to challenge fibrogenesis as a means to combat this progressive and fatal disease.

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