Title

C-C motif chemokine ligand 15 may be a useful biomarker for predicting the prognosis of patients with chronic hypersensitivity pneumonitis

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Short title

CCL15 may be a useful biomarker for predicting the prognosis of CHP.

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1. Abstract

Background: Chronic hypersensitivity pneumonitis (CHP) is characterized by lymphocytic inflammation and progressive fibrosis of the lung caused by a variety of inhaled antigens. Due to the difficulty of accurately diagnosing CHP, and the poor prognosis associated with the condition, a novel clinical biomarker is urgently needed.

Objective: To investigate the usefulness of C-C motif chemokine ligand 15 (CCL15), which had been demonstrated to highly express in the lungs of CHP patients, as a clinical biomarker for CHP.

Method: Immunohistochemical investigations were performed on lung tissue from CHP patients, and CCL15 levels in serum and bronchoalveolar lavage fluid (BALF) were measured via the enzyme-linked immunosorbent assay.

Results: Immunohistochemistry investigations revealed high CCL15 expression in the lungs of CHP patients. Serum CCL15 levels in CHP patients (29.1 ± 2.1 μg/ml) were significantly higher than those of idiopathic pulmonary fibrosis patients (19.7 ± 1.3 μg/ml, $p = 0.01$) and healthy subjects (19.5 ± 1.7 μg/ml, $p = 0.003$). When BALF CCL15 level was divided by BALF albumin level (BALF CCL15/Alb), it was significantly inversely correlated with forced vital capacity (β = -0.47, $p = 0.0006$), percentage of predicted carbon monoxide diffusion capacity of the lung (β = -0.41, $p = 0.0048$), and BALF lymphocyte count (β = -0.34, $p = 0.01$) in CHP patients. Multivariate Cox proportional hazards analysis revealed that high BALF CCL15/Alb and poor prognosis were statistically significantly independently correlated in CHP patients (HR = 1.1, 95% confidence interval 1.03–1.18, $p = 0.004$).

Conclusion: The results of the current study suggest that CCL15 may be a useful prognostic biomarker for CHP. CCL15 was highly expressed in the lung tissue of CHP patients, and BALF CCL15/Alb was significantly associated with CHP prognosis.

2. Introduction

Chronic hypersensitivity pneumonitis (CHP) is characterized by lymphocytic inflammation and progressive fibrosis predominantly involving the lung interstitium caused by a broad range of inhaled antigens [1-3]. Centrilobular nodules predominantly distributed in the upper lobes accompanied by reticular shadows with or without honeycombing are typical findings in high-resolution computed tomography (HRCT) of CHP patients [4, 5].

Despite the features described above, accurate diagnosis of CHP is often difficult for several reasons. The identification of causative inhaled antigens requires careful and detailed interviewing. Second, radiological findings of CHP often mimic those of idiopathic pulmonary fibrosis (IPF) or other fibrosing interstitial pneumonias [3, 6]. Third, there are no established diagnostic criteria for CHP that are applied worldwide. Nonetheless, accurate and prompt diagnosis is of critical importance, because rapid deterioration of pulmonary function occurs in some patients and is associated with a poor prognosis [7-11], and therapeutic strategies for CHP differ from those for other fibrosing interstitial pneumonias. Avoidance or minimization of exposure to the causative antigen is the basic principle in the management of CHP [3]. In contrast, anti-inflammatory or anti-fibrotic agents should be used in patients with other fibrosing interstitial pneumonias. Thus, there is a strong need for a novel clinical biomarker to facilitate accurate diagnosis and predict outcomes of CHP patients.

We recently performed gene expression analysis using surgically resected lung tissue from 9 CHP patients [12]. We identified several upregulated genes in diseased lungs compared to control lungs. Among them, we focused on C-C motif chemokine ligand 15 (CCL15), a secreted chemoattractant protein belonging to the macrophage inflammatory protein-1 family [13], because CCL15 mRNA expression was 20-fold higher or greater in the lungs of 6 of the 9 CHP patients than in the lungs of the controls [12]. Interestingly, we also found that the ratio of CCL15 mRNA expression in the lung tissue of IPF patients to that of control lung tissue was

less than 0.05 in 6 out of 7 IPF patients in another gene expression analysis [14]. Based on these contrasting mRNA expression profiles, we speculated that the involvement of CCL15 in the pathogenesis of interstitial inflammation and/or fibrosis in CHP may differ from that of IPF. Thus, CCL15 may be a useful biomarker for CHP.

Based on the afore-mentioned findings and speculations, we designed the present study to investigate the usefulness of CCL15 as a clinical biomarker for CHP.

3. Materials and Methods

Subjects

The subjects enrolled in the study were 51 Japanese patients with CHP, 78 with IPF, and 69 healthy control subjects. All CHP and IPF patients were diagnosed at Hiroshima University Hospital between 2001 and 2014. Diagnoses of CHP and IPF were made in accordance with the diagnostic criteria for CHP proposed by Yoshizawa and coworkers in 1999 [1] and the ATS/ERS/JRS/ALAT joint statement for IPF in 2011 [15], respectively. Briefly, CHP is diagnosed when three or more of the following conditions [including either i) or ii), iii) or iv), and v) or vi)] are fulfilled: i) recurrence of symptoms by environmental provocation or antigen inhalation, ii) presence of antibodies and/or lymphocytic proliferation targeting the specific antigen, iii) evidence of pulmonary fibrosis with or without granulomas in histopathological analysis, iv) honeycomb pattern on CT, v) progressive deterioration of restrictive pulmonary function impairment over 1 year, and vi) persistence of respiratory symptoms related to the disease for more than 6 months [1]. We also investigated whether the patients with CHP enrolled in this study fulfilled the newer diagnostic criteria proposed by Vasakova and coworkers [16].

The healthy subjects were recruited from a pool of recipients of health checkups at the hospital consisting of a disease screening questionnaire, chest X-ray, pulmonary function tests, and electrocardiography. Those with apparent lung diseases such as COPD or interstitial lung diseases were excluded. Additionally, those with lung cancer, colon cancer, liver cancer, asthma, or chronic renal failure were also excluded from both patient groups and the control group, because these diseases have been reported to be associated with increased expression of CCL15 [17-24]. The present study was approved by the Ethics Committee of Hiroshima University Hospital (approval number 326) and conducted in accordance with the ethical standards established in the Helsinki Declaration of 1975. All participants provided written informed

consent to use their samples for this study and to publish the results.

Serum and bronchoalveolar lavage sampling procedure

Serum samples were obtained from blood drawn during initial patient assessments or during health checkups. Freshly acquired blood samples were promptly centrifuged and cryopreserved at -80℃ until they were analyzed. Bronchoalveolar lavage (BAL) was performed under local anesthesia, by introducing 50 mL of saline into the lung and promptly drawing it out again via a bronchoscope. These procedures were repeated three times and 150mL of saline in total was introduced during BAL. The BAL target region was either the right middle lobe or the lingular segment of left lung, depending on which was the most severely affected as determined in accordance with the findings of HRCT. The retrieved saline was centrifuged promptly, and the supernatant was cryopreserved at -80℃ until it was analyzed.

Pulmonary function tests

Spirometry and carbon monoxide diffusion capacity of the lung (DLco) measurements were performed in accordance with the recommendations of the American Thoracic Society [25]. Annual declines in forced vital capacity (FVC) and DLco were calculated by dividing the slope of the regression lines for FVC or DLco by the baseline values, as previously described [12].

Immunohistochemistry staining for CCL15

Lung tissue sections from CHP patients and IPF were obtained from surgical lung biopsy specimens that had been acquired for diagnostic purposes. Control lung tissue sections were obtained from the healthy areas of lungs that had been surgically resected along with lung tumors for therapeutic purposes. These lung tissue sections were stained using the ENVISION+

Kit/horseradish peroxidase (HRP) (Dako, Tokyo, Japan) as previously described [14]. Anti-CCL15 antibody (1:300, Lot ID: 52039; LifeSpan BioSciences, Inc., Seattle, WA, USA) was added after blocking of endogenous peroxidase and proteins. The sections were then incubated with HRP-labeled anti-goat IgG secondary antibody followed by the addition of substrate-chromogen and counterstaining with hematoxylin.

Measurement of CCL15 and albumin concentrations

CCL15 levels in serum and BAL fluid (BALF) were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). BALF albumin levels were measured using a commercially available immunoturbidity kit (AutoWako Microalbumin; Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis

For numerical variables, means ± the standard error of the mean (SEM) were calculated. Either the Mann-Whitney *U* test or Pearson's chi-square test were used to assess comparisons between groups, as appropriate. Receiver operating characteristic (ROC) curve analysis was performed to determine the appropriate CCL15 threshold for predicting 5-year survival. The difference in survival between the two groups was evaluated using the log-rank test. The predictive ability of CCL15 was tested via a Cox proportional hazards regression model. All data analyses were performed with JMP PRO statistical software version 12.2.0 (SAS Institute Inc., Cary, NC, USA).

4. Results

Baseline characteristics

The baseline characteristics of the 51 patients with CHP, 78 patients with IPF, and 69 healthy subjects included in the current study are shown in Table 1. Among these subjects, serum samples were available for 46 of the 51 patients with CHP, all 78 patients with IPF, and all 69 healthy subjects. BALF samples were available for all 51 patients with CHP, and 53 of the 78 patients with IPF. Compared to the IPF group, the CHP group contained less male subjects, and the subjects had lighter smoking histories and more lymphocytes in BALF.
As shown in Supplementary Table 1, there were no significant differences in the baseline characteristics of the serum analysis groups and the BALF analysis groups for CHP or IPF.

Diagnosis of CHP

As described above, all patients with CHP fulfilled the diagnostic criteria proposed by Yoshizawa and coworkers in 1999 [1]. According to Vasakova's criteria [16], 20, eight, and 23 patients exhibited "confident clinically diagnosed HP", "probable HP," and "possible HP," respectively. Seven of the eight patients with "probable HP" and 22 of the 23 patients with "possible HP" showed pathological findings compatible with HP. Therefore, 49 of the 51 patients with CHP were considered to have "confident clinically diagnosed HP" and/or "definite HP" on the basis of Vasakova's criteria. As shown in Table 1, specific immunoglobulin (Ig) G or IgA for pigeon, parrot, budgerigar, and/or *Trichosporon asahi* was detected in 21 patients with CHP.

CCL15 expression is increased in the lungs of CHP patients

Immunohistochemistry results revealed that CCL15 was markedly expressed in plasma cells and macrophages infiltrating the fibrosing interstitium and macrophages in the alveolar

space in the lungs of CHP patients (Figure 1a, 1b). Week CCL15 expression was detected in the interstitial plasma cells, and macrophages in the lungs of IPF patients (Figure 1c). CCL15 expression was not detected in the control lungs (Figure 1d).

Serum CCL15 are significantly higher in CHP patients than in IPF patients and healthy controls

Serum CCL15 in CHP patients (29.1 ± 2.1 μg/ml) were significantly higher than those in IPF patients (19.7 ± 1.3 μg/ml; $p = 0.01$) and healthy subjects (19.5 ± 1.7 μg/ml; $p = 0.003$) (Figure 2a). In CHP patients, BALF CCL15 tended to be higher than they were in IPF patients, although the difference was not statistically significant (Figure 2b).

BALF CCL15 was correlated with baseline pulmonary function and with serial change in pulmonary function

To assess the role of CCL15 in the development of clinical manifestations of CHP, we investigated whether serum or BALF CCL15 were significantly correlated with clinical characteristics including pulmonary function and BALF cell properties. As shown in Table 2, neither serum nor BALF CCL15 were significantly correlated with pulmonary function or BALF total cell or lymphocyte count, whereas there was a weak correlation between BALF CCL15 and FVC ($p = 0.02$). Importantly, when divided by BALF albumin (Alb) level, BALF CCL15 was significantly correlated with FVC ($p < 0.001$), percentage of predicted forced vital capacity (%FVC) ($p = 0.002$), DLco ($p = 0.002$), and percentage of predicted carbon monoxide diffusion capacity of the lung (%DLco) ($p = 0.005$) (Table 2). BALF CCL15 was also only correlated with BALF lymphocyte counts and BALF lymphocyte fractions when it was divided by BALF Alb ($p = 0.01$ and $p = 0.005$, respectively, Table 2). Higher BALF CCL15/BALF Alb (BALF CCL15/Alb) was significantly correlated with more severe annual decline in FVC in

CHP patients (p = 0.0024, Figure 3). Conversely, neither serum CCL15 nor BALF CCL15 was significantly correlated with pulmonary function or BALF cell properties in IPF patients (Supplementary Table 2).

BALF CCL15/Alb can predict CHP prognosis

Based on the above results, we surmised that BALF CCL15/Alb may be a useful biomarker for CHP. This method has previously been utilized to remove the variable of dilution, and also to remove the influence of serum protein that has infiltrated into the alveolar space from capillary vessels [26]. On the basis of ROC curve analysis, we determined that the appropriate BALF CCL15/Alb threshold for predicting 5-year survival in patients with CHP was 10.82. As shown in Supplementary Figure 1, the survival rate for patients with a high BALF CCL15/Alb was significantly lower than that for patients with a low BALF CCL15/Alb (P = 0.002). To further investigate the prognostic and predictive capacities of BALF CCL15/Alb in CHP patients, we performed Cox proportional hazards analysis. As shown in Table 3, high BALF CCL15/Alb, impaired pulmonary function, and a low lymphocyte count in BALF were significantly correlated with a poor prognosis in CHP patients. However, neither serum nor BALF CCL15 were significantly correlated with prognosis (Table 3). Multivariate analysis including BALF CCL15/Alb, FVC, and BALF lymphocyte counts as covariates revealed that the correlation between high BALF CCL15/Alb and poor prognosis was statistically independent in CHP patients (p = 0.004, Table 3). BALF CCL15/Alb was not correlated with prognosis in IPF patients (Supplementary Table 3).

5. Discussion

The most important finding in the present study was that BALF CCL15/Alb was significantly correlated with prognosis of patients with CHP. However, neither serum nor BALF CCL15 were significantly correlated with prognosis. These results suggest that lung locally-derived CCL15 may play an important role in the pathogenesis of CHP. To the best of our knowledge, this is the first report indicating the usefulness of CCL15 as a prognostic biomarker for CHP. In the present study, the expression of CCL15 protein was increased in the lungs of CHP patients. This is consistent with the previously reported increased expression of CCL15 mRNA in the lungs of CHP patients [12].

Higher BALF CCL15/Alb was significantly associated with poorer pulmonary functions at baseline, and with a greater decline in pulmonary function over time. As it has been previously reported that the major biological function of CCL15 involves chemotactic activity that attracts T-lymphocytes, monocytes, and vascular endothelial cells, mainly via CC chemokine receptor (CCR) 1 or CCR3 [27, 28], these pathways may play an important role in the pathogenesis of CHP. CCR1 knockdown has been reported to reduce the secretion of matrix metalloproteinase (MMP)-2 or MMP-9 by the several types of cancer [29, 30]. Increased CCR1 expression by neutrophils, monocytes, eosinophils, dendritic cells, activated T cells, and B lymphocytes is also known to be associated with autoimmune diseases such as multiple sclerosis and rheumatoid arthritis [13, 30]. These findings suggest that CCL15 may promote inflammatory change in the lungs of CHP patients via interaction with CCR1. These speculations correspond with the results of a previous study involving pathway analysis in CHP, in which we demonstrated that several pathways related to inflammatory responses and immunological diseases were differentially expressed in patients with rapidly progressive CHP [12].

CCR3 is known to be expressed on eosinophils, basophils, mast cells, and Th2 cells [30,

31]. Interestingly, other Th2-related chemokines including CCL17 and CCL18 have been reported to be involved in the progression of fibrosis [33-35]. Therefore, we can speculate that CCL15 plays some role in lung fibrosis through its interaction with CCR3.

CCL15 was only significantly correlated with baseline pulmonary function, functional deterioration, and prognosis after it was divided by BALF Alb. CCL15 is not a lung-specific protein, but it is widely expressed in various organs [36-38]. Thus, BALF CCL15 may not only reflect locally-derived CCL15, but also systemically produced CCL15 that is present due to capillary-alveolar permeability. In addition, BALF protein levels measured by ELISA can be influenced by the amount of injected saline as well as the amount of recovered saline [39]. In order to negate the influence of systemically produced CCL15 infiltrating the alveolar space and also to remove the variable of dilution inherently associated with the BAL procedure, we divided BALF CCL15 concentration by BALF Alb concentration [26, 40-42]. As BALF CCL15/Alb is considered to reflect locally derived lung CCL15, these results suggest that locally derived lung CCL15, not systemically-produced CCL15, has an important role in the pathogenesis of CHP and that it can be a useful prognostic biomarker in CHP.

Serum CCL15 were significantly higher in CHP patients than in IPF patients. These results are concordant with the differential expression of CCL15 mRNA in the lungs of CHP patients and IPF patients. It may be that CCL15 is inherently involved in the pathogenesis of CHP, whereas its involvement in the pathogenesis of IPF is limited. Notably, the prognostic predictive capacity of BALF CCL15 was significant in CHP patients but not in IPF patients.

The current study had several limitations. The sample size was limited because the study was conducted at a single facility. The study design was also retrospective, and there was no validation cohort. Thus, multicenter prospective studies are needed to confirm the usefulness of CCL15 as a prognostic biomarker in CHP. Despite these limitations, we believe the present study attests to the prognostic predictive capacity of CCL15 in CHP patients.

Conclusion

The results of the present study suggest that CCL15 may be a useful prognostic biomarker in CHP.

8.1 Acknowledgement

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8.2 Statement of Ethics

The manuscript has not been published or presented elsewhere in part or in entirety, and it is not under consideration by another journal. All study participants provided informed consent, and the study design was approved by the appropriate ethics review board. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these.

8.3 Disclosure Statement

The authors have no conflicts of interest to declare.

8.4 Funding Sources

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8.5 Author Contributions

Masako Watanabe drafted and finalized the manuscript, performed part of the serum measurement, immunohistochemistry staining and statistical analyses. Yasushi Horimasu, Hiroshi Iwamoto, Hironobu Hamada, Nobuoki Kohno and Noboru Hattori conceived the study, and participated in its design and coordination and helped to draft and finalize the manuscript. Kakuhiro Yamaguchi performed part of the serum measurement and statistical analysis. Shinjiro Sakamoto, Takeshi Masuda, Taku Nakashima, Shintaro Miyamoto, Shinichiro Ohshimo and Kazunori Fujitaka recruited the study subjects, ascertained diagnosis, and helped to draft and finalize the manuscript. All authors read and approved the final manuscript.

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10. Figure Legends

Fig. 1. Immunohistochemistry staining for CCL15 in CHP, IPF, and control lungs. Scale bar = 100 μm. CCL15-positive cells are indicated by black arrows. a) CHP. b) High-power field in CHP. c) IPF. d) Control.

Fig. 2. a) In CHP patients, serum CCL15 were significantly higher than they were in IPF patients and healthy subjects. b) In CHP patients, BALF CCL15 tended to be higher than they were in IPF patients ($p = 0.06$, Mann-Whitney *U* test).

$^{*}p = 0.003$, $^{**}p = 0.01$, Mann-Whitney *U* test with Bonferroni correction

Fig. 3. High BALF CCL15/Alb was significantly associated with change in FVC in CHP patients.

$\beta = -0.47$, $p = 0.0024$, linear regression analysis

Table 1 Baseline patient characteristics.

	CHP	IPF	Control
Sample *n*	51	78	69
Serum/BALF available	46/51	78/53	69/0
Male/female	29/22	69/9	59/10
Age in years	68.4 ± 1.2	68.9 ± 1.0	62.0 ± 0.9
Smoking history (yes/no)	28/23	66/12	54/15
Pulmonary function tests			
FVC (l)	2.2 ± 0.1	2.5 ± 0.1	3.5 ± 0.1
%FVC (%)	75 ± 3.4	74.5 ± 2.4	98.8 ± 2.4
DLco (ml/min/mmHg)	10.2 ± 0.7	10.8 ± 0.5	NA
%DLco (%)	47.7 ± 2.6	46.3 ± 1.9	NA
BALF			
Total cell (10^4/ml)	31.4 ± 3.8	21.3 ± 2.1	NA
Lymphocyte (10^4/ml)	9.9 ± 2.2	2.3 ± 0.5	NA
Lymphocyte (%)	28.2 ± 3.2	10.4 ± 1.2	NA
Biopsy, VATS/TBLB/NA	19/29/3	14/33/31	0/0/69
Antibodies, Avian/*T, asahi*/NA	13/8/30	0/0/78	0/0/69

Numerical data are represented as mean ± the standard error of the mean.

CHP, chronic hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis; BALF, bronchoalveolar lavage fluid; FVC, forced vital capacity; %FVC, percentage of predicted forced vital capacity; DLco, carbon monoxide diffusion capacity of the lung; %DLco, percentage of predicted carbon monoxide diffusion capacity of the lung; VATS, Video- assisted thoracic surgery: TBLB, Transbronchial lung biopsy: Avian, Avian specific antibodies includes

IgG or IgA for pigeon, parrot or budgerigar: *T,asahi,* anti- *Trichosporon asahii* antibody: NA, not assessed.

Table 2 Correlations between CCL15 and baseline characteristics in chronic hypersensitivity pneumonitis patients.

	Serum CCL15 (n = 46)			BALF CCL15 (n = 51)			BALF CCL15/Alb (n = 51)		
	t	β	p value	t	β	p value	t	β	p value
Gender (male)	0.46	0.07	0.65	1.19	0.17	0.24	0.95	0.13	0.35
Age (years)	-2.06	-0.3	0.046*	0.35	0.05	0.73	1.04	0.15	0.3
Smoking history (yes)	-0.85	-0.14	0.4	-0.69	-0.1	0.49	-0.77	-0.11	0.44
BALF CCL15 (μg/ml)	1.76	0.26	0.09	-	-	-	-	-	-
BALF CCL15/Alb	0.02	0.003	0.99	-	-	-	-	-	-
FVC (l)	-0.77	-0.12	0.45	-2.52	-0.35	0.02*	-3.69	-0.47	0.0006*
%FVC (%)	-1	-0.17	0.32	-1.58	-0.25	0.12	-3.39	-0.48	0.0016*
DLco (ml/min/mmHg)	-1.61	-0.26	0.12	-1.58	-0.24	0.12	-3.38	-0.47	0.0016*
%DLco (%)	-1.48	-0.23	0.15	-0.73	-0.11	0.47	-2.97	-0.41	0.0048*
BALF total cell (10^4/ml)	-0.51	-0.08	0.61	-0.61	-0.09	0.54	-0.98	-0.14	0.33
BALF lymphocyte (10^4/ml)	0.1	0.02	0.92	-0.2	-0.03	0.84	-2.55	-0.34	0.01*
BALF lymphocyte (%)	1.41	0.23	0.17	0.51	0.07	0.61	-2.96	-0.39	0.005*

CCL15, C-C motif chemokine ligand 15; BALF, bronchoalveolar lavage fluid; Alb, albumin; FVC, forced vital capacity; %FVC, percentage of

predicted forced vital capacity; DLco, carbon monoxide diffusion capacity of the lung; %DLco, percentage of predicted carbon monoxide diffusion capacity of the lung

*$p < 0.05$, linear regression analysis

Table 3 Univariate and multivariate Cox proportional hazards regression model in chronic hypersensitivity pneumonitis patients.

	Univariate			Multivariate		
	HR	95% CI	*p*	HR	95% CI	*p*
Serum CCL15, continuous	1.00	0.95–1.04	0.96			
BALF CCL15, continuous	2.14	0.83–4.82	0.11			
BALF CCL15/Alb, continuous	1.12	1.06–1.19	0.0001*	1.10	1.03–1.18	0.004*
Gender (male)	0.86	0.30–2.63	0.78			
Age (years)	1.02	0.96–1.09	0.54			
Smoking history (yes)	0.56	0.19–1.64	0.28			
FVC, continuous	0.42	0.17–0.98	0.04*	0.76	0.27–2.13	0.59
%DLco, continuous	0.96	0.92–0.99	0.02*			
BALF total cell, continuous	1.00	0.98–1.02	0.68			
BALF lymphocyte, continuous	0.92	0.78–1.00	0.04*	0.96	0.82–1.03	0.36

HR, hazard ratio; CI, confidence interval; CCL15, C-C motif chemokine ligand 15; BALF, bronchoalveolar lavage fluid; Alb, albumin; FVC, forced vital capacity; %DLco, percentage of predicted carbon monoxide diffusion capacity of the lung

*$p < 0.05$, Cox proportional hazards regression model

Supplementary Figure 1 Kaplan–Meier curves demonstrating the survival rate for patients with CHP stratified according to the BALF CCL15/Alb. The solid line represents patients with a low BALF CCL15/Alb (n = 33), while the dotted line represents patients with a high BALF/Alb (n = 18) . BALF, bronchoalveolar lavage fluid; Alb, albumin.

*p = 0.002, log-rank test.

Supplementary Table 1 Baseline patient characteristics.

	CHP		IPF	
	BALF	Serum	BALF	Serum
Sample (n)	51	46	53	78
Gender (Male/female)	29/22	26/20	49/4	69/9
Age (year)	68.4±1.2	69.3±1.2	67.1±1.1	68.9±1.0
Smoking history (yes/no)	28/23	25/21	46/7	66/12
Pulmonary function tests				
FVC (l)	2.2±0.1	2.1±0.1	2.5±0.1	2.5±0.1
%FVC (%)	75±3.4	72.7±3.6	74.8±2.8	74.5±2.4
DLco (ml/min/mmHg)	10.2±0.7	9.5±0.7	11±0.6	10.8±0.5
%DLco (%)	47.7±2.6	45.2±2.4	47.1±2.3	46.3±1.9
BALF				
Total cell ($\times 10^4$/ml)	31.4±3.8	29.6±3.7	20.5±2.1	21.3±2.1
Lymphocyte ($\times 10^4$/ml)	9.9±2.2	9.7±2.3	2.1±0.5	2.3±0.5
Lymphocyte (%)	28.2±3.2	28.2±3.3	10.1±1.2	10.4±1.2

Numerical data are represented as mean ± the standard error of the mean.

CHP, chronic hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis; BALF, bronchoalveolar lavage fluid; FVC, forced vital capacity; %FVC, percentage of predicted forced vital capacity; DLco, carbon monoxide diffusion capacity of the lung; %DLco, percentage of predicted carbon monoxide diffusion capacity of the lung.

Supplementary Table 2 Correlations between CCL15 and baseline characteristics in idiopathic pulmonary fibrosis patients.

	Serum CCL15 (*n* = 78)			BALF CCL15 (*n* = 53)			BALF CCL15 /Alb (*n* = 53)		
	t	β	*p* value	t	β	*p* value	t	β	*p* value
Gender (male)	0.17	0.02	0.87	-1.68	-0.23	0.2	-0.87	-0.12	0.39
Age (year)	1.18	0.13	0.24	-0.53	-0.07	0.6	-0.93	-0.13	0.35
Smoking history (yes)	0.17	0.02	0.87	-1.09	-0.15	0.28	-3.17	-0.41	0.003*
BALF CCL15 (μg/ml)	-1.11	-0.15	0.27	-	-	-	-	-	-
BALF CCL15/Alb	-0.67	-0.09	0.51	-	-	-	-	-	-
FVC (l)	0.61	0.08	0.54	0.5	0.07	0.62	0.81	0.12	0.42
%FVC (%)	1.46	0.19	0.15	-0.36	-0.05	0.72	-0.18	-0.03	0.86
DLco (ml/min/mmHg)	0.1	0.01	0.92	-0.29	-0.04	0.78	-0.5	-0.08	0.62
%DLco (%)	-0.02	-0.002	0.98	-0.51	-0.07	0.62	-0.91	-0.13	0.37
BALF total cell (10^4/ml)	-0.23	-0.03	0.82	-0.92	-0.13	0.36	-0.87	-0.12	0.39
BALF lymphocyte (10^4/ml)	0.63	0.09	0.53	-1.53	-0.21	0.13	-1.53	-0.21	0.13
BALF lymphocyte (%)	1.56	0.21	0.13	-2.01	-0.27	0.05	-1.51	-0.21	0.14

CCL15, C-C motif chemokine ligand 15; BALF, bronchoalveolar lavage fluid; Alb, albumin; FVC, forced vital capacity; %FVC, percentage of predicted forced vital capacity; DLco, carbon monoxide diffusion capacity of the lung; %DLco, percentage of predicted carbon monoxide diffusion capacity of the lung.

$^{*}p < 0.05$, linear regression analysis

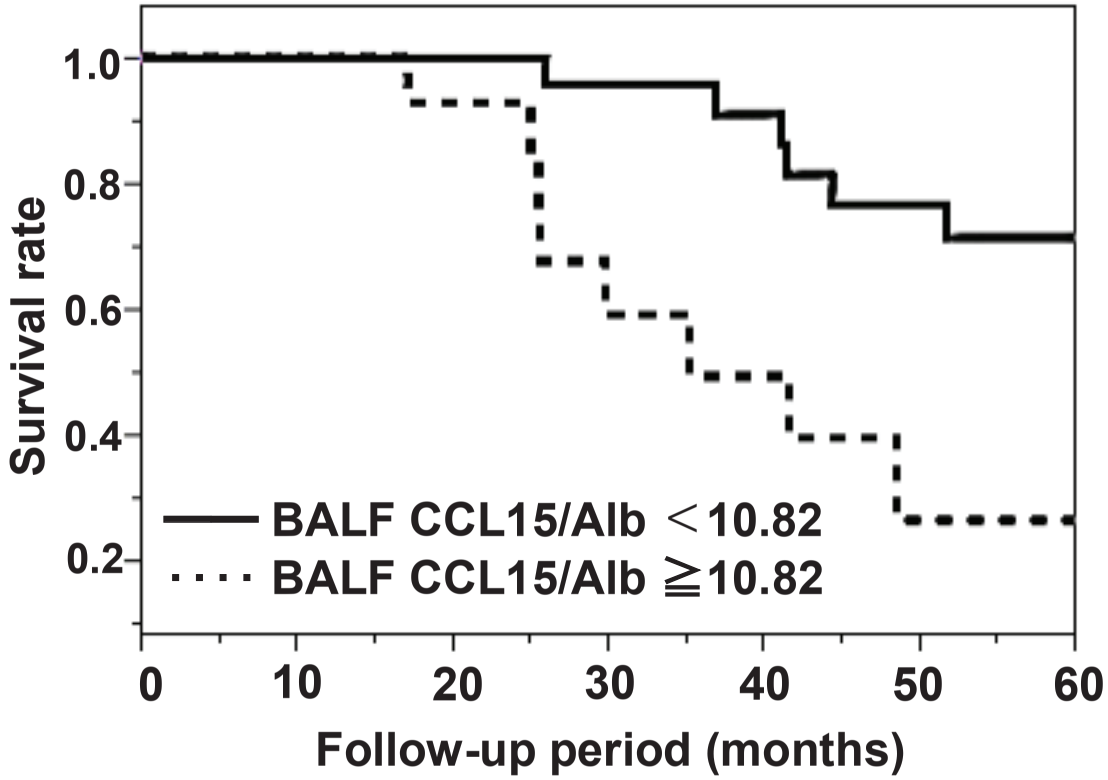
Supplementary Table 3 Univariate Cox proportional hazard regression model in idiopathic pulmonary fibrosis patients.

	HR	95% CI	*p*
Serum CCL15, continuous	1.01	0.97-1.06	0.52
BALF CCL15, continuous	1.33	0.3-4.57	0.69
BALF CCL15/Alb, continuous	1.02	0.99-1.044	0.28
Gender, male	0.59	0.17-3.70	0.52
Age (years)	0.98	0.92-1.03	0.42
Smoking history, yes	0.43	0.14-1.91	0.24
FVC, continuous	0.58	0.27-1.13	0.11
%DLco, continuous	0.96	0.93-0.99	0.02*
BALF total cell, continuous	0.96	0.91-0.99	0.04*
BALF lymphocyte, continuous	0.78	0.48-1.00	0.04*

HR, hazard ratio; CI, confidence interval; IPF, idiopathic pulmonary fibrosis; CCL15, C-C motif chemokine ligand 15; BALF, Bronchoalveolar Lavage Fluid**;** FVC, forced expiratory volume; DLco, diffusing capacity of the lung for carbon monoxide;

*$p < 0.05$, Cox proportional hazard regression model.

Supplementary Figure 1



Number at risk							
BALF CCL15/Alb ＜10.82	33	28	25	23	19	15	11
BALF CCL15/Alb ≧10.82	18	15	13	8	6	2	1

Figure 1

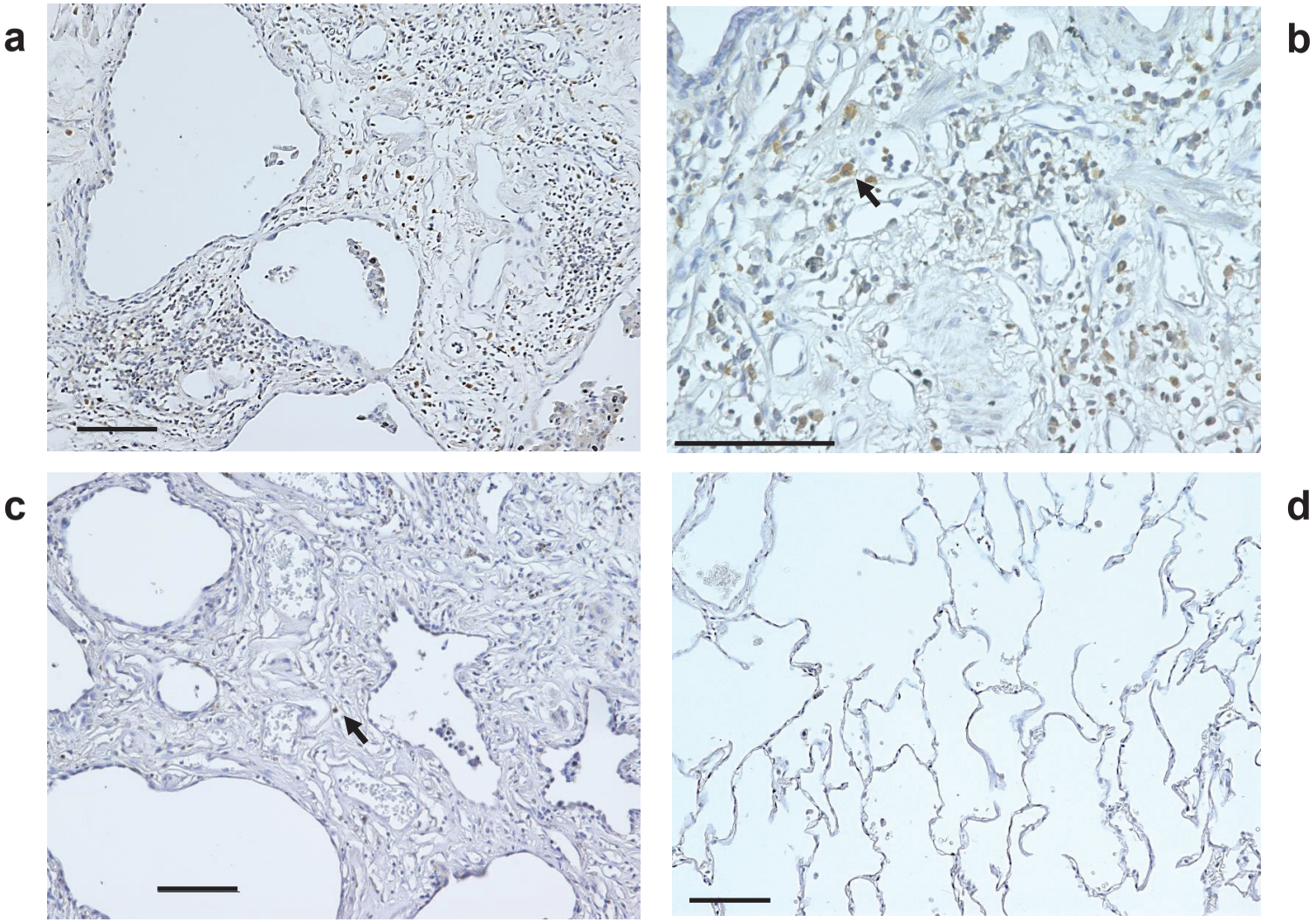
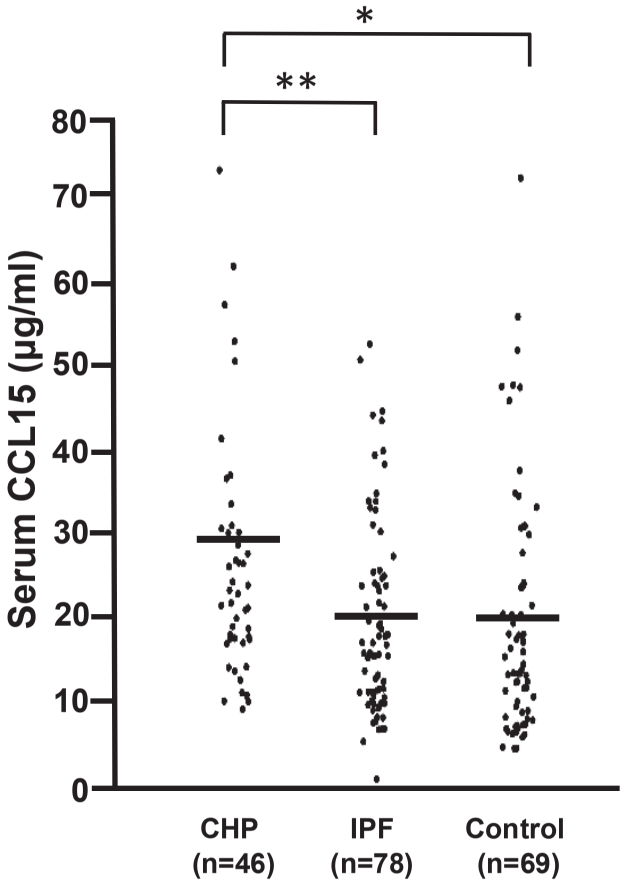
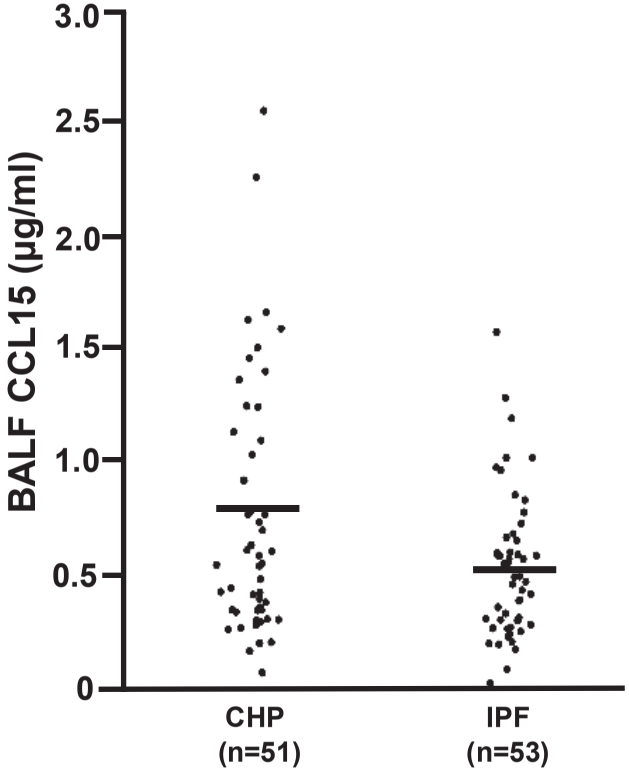


Figure 2a



	CHP (n=46)	IPF (n=78)	Control (n=69)
mean ± SEM	29.1 ± 2.1	19.7 ± 1.3	19.5 ± 1.7

Figure 2b



	CHP (n=51)	IPF (n=53)
mean ± SEM	0.76 ± 0.08	0.54 ± 0.04

Figure 3