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Original Article

Acute inflammatory and immunologic responses against antigen in chronic bird-related hypersensitivity pneumonitis

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BALF, bronchoalveolar lavage fluid; BRHP, bird-related hypersensitivity pneumonitis; CRP, C reactive protein; DLco, diffusing capacity for carbon monoxide; G-CSF, granulocyte colony-stimulating factor; HP, hypersensitivity pneumonitis; ILDs, interstitial lung diseases; IPF, idiopathic pulmonary fibrosis; IPT, inhalation provocation test; KL-6, Krebs von den Lungen-6; LDH, lactate dehydrogenase; LPT, lymphocyte proliferation test; PDE, pigeon dropping extracts; SI, stimulation index; SP-D, surfactant protein D; VC, vital capacity; WBC, white blood cell

ABSTRACT

Background: Hypersensitivity pneumonitis (HP) is an immune-mediated lung disease induced by the inhalation of a wide variety of antigens and a persistent antigen exposure induces inevitably pulmonary fibrosis in chronic HP. Although neutrophils, Th1 and Th17 cells contribute to lung inflammation in acute phase of HP, there is no clear explanation as to how the immunological reaction occurs just after the inhalation of causative antigens in the chronic phase of HP.

Methods: We examined the inflammatory and immunologic profiles before and after the inhalation provocation test (IPT) in serum and bronchoalveolar lavage fluid (BALF) from patients with chronic bird-related HP (BRHP) and other interstitial lung diseases (ILDs). We analyzed BALF samples from 39 patients (19 BRHP and 20 other ILDs) and serum samples from 25 consecutive patients (20 BRHP and 5 other ILDs) who underwent the IPT.

Results: A significant increase of neutrophils was observed in the BALF from the BRHP patients following the IPT. Neutrophil chemoattractants, namely, granulocyte colony-stimulating factor, IL-6, IL-8, IL-17, and CXCL2 significantly increased in both the serum and BALF of the BRHP patients after the IPT. Serum IFN- γ and CXCL10, cytokines/chemokines that contributed to Th1 inflammation, were also significantly increased in BRHP following the IPT.

Conclusions: This study demonstrated the exposure to the causative antigen provoked acute neutrophilic and Th1 immunologic responses similar to acute HP even in the chronic phase of HP.

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Introduction

Hypersensitivity pneumonitis (HP) is an immune-mediated lung disease induced by the inhalation of various antigens.¹ HP is classified into acute and chronic forms,² the pathogenesis of which is induced by the formation of immune complex

by IgG-class-specific antibodies and/or a T cell hypersensitivity mechanism following the inhalation of specific antigens.³ Increases in neutrophils can be found in bronchoalveolar lavage fluid (BALF) in the early phase after exposure to causative antigens.^{4–7}

Two studies have investigated the roles of cytokines and chemokines in acute and chronic HP.^{8,9} IL-8 and monocyte chemoattractant protein-1/C-C motif ligand 2 (MCP-1/CCL2) are significantly elevated in acute HP.⁸ Both cytokines/chemokines are significantly correlated with the number of BALF lymphocytes in acute HP patients, and IL-8 is also correlated with the number of BALF neutrophils.⁸ IFN- γ and IFN- γ -inducible T cell

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protein 10/C-X-C motif ligand 10 (IP10/CXCL10), the Th1 cytokines/chemokines, are both known to contribute to granuloma formation and the induction of alveolitis.⁹ Our group, meanwhile, found that Th2 immune response was predominant in chronic HP with fibrosis and that the Th1/Th2 balance shifted to Th2 predominance as pulmonary fibrosis progressed.¹⁰ Serum CCL17 (Th2 chemokine) was significantly higher in patients with bird-related hypersensitivity pneumonitis (BRHP) with usual interstitial-pneumonia-like lesions than in BRHP patients with less pulmonary fibrosis. Elevated serum levels of CCL17 have also been identified as a risk factor of acute exacerbation of chronic BRHP.¹¹

BRHP, one of the common types of HP, is induced by exposure to avian antigens such as pigeon droppings or feathers.¹² A nationwide epidemiological survey identified BRHP as the most prevalent form of chronic HP in the Japanese population.¹³ The clinical features of chronic HP are similar to those of idiopathic pulmonary fibrosis (IPF), which often makes it difficult to distinguish between the two diseases.¹⁴ Because precipitating antibody can be detected in serum with low sensitivity (about 30%) in chronic BRHP,¹⁵ the inhalation provocation test (IPT) is now considered the gold standard for diagnosing that disease.^{6,16–18}

As of this writing, however, we have no clear picture of the mechanisms responsible for the immunological reaction induced just after a patient with chronic BRHP inhales causative antigens. Reynolds SP *et al.* found no significant difference between the pre- and post-IPT levels of IL-1 or IL-2 in serum from patients with acute BRHP.¹⁹ Villar A *et al.*, in contrast, observed higher sputum eosinophils and a tendency towards increased Th2 cytokine (IL-4, IL-5) following specific inhalation challenge in patients with BRHP versus those with HP caused by fungi.²⁰ An evaluation of cytokines and chemokines during the IPT in BRHP patients may help elucidate the pathophysiology of BRHP and discriminate between the acute and chronic variants.

We performed this study to elucidate the inflammatory and immunological profiles in serum and BALF from patients with chronic BRHP before and after the IPT and to elucidate the immunological reactions involved after the inhalation of specific antigens in the chronic phase of BRHP.

Methods

Study population

The clinical records of 755 patients with interstitial lung diseases (ILDs) admitted to the Tokyo Medical and Dental University Hospital between April 2000 and March 2011 were examined in a retrospective sequential review. Among the patients who underwent the IPT using avian dropping extract, 39 patients who underwent BAL before and after the IPT were extracted (Fig. 1A). We then performed prospective cohort consisted of 27 consecutive patients who underwent the IPT between April 2013 and March 2018 to evaluate systemic inflammation after the IPT. Serum samples were obtained both before and after (6 and 24 h) the IPT in 25 of the 27 patients prospectively studied (Fig. 1B). Informed consent was obtained from all of the patients, and the study was approved by the institutional review board at the Tokyo Medical and Dental University (approval numbers: M2000-1496, 1497, M2016-176).

Diagnostic criteria for chronic HP

Yoshizawa's criteria was used for the diagnosis of chronic HP, as previously described.²¹ The diagnostic criteria were three or more of the following (including either 1 or 2, either 3 or 4, and either 5 or 6): (1) reproduction of symptoms of HP by an environmental or inhalation provocation test, (2) the presence of specific antibodies and/or positive lymphocyte proliferative reactions to avian antigen, (3) evidence of pulmonary fibrosis with or without granulomas in histology, (4) a finding of honeycombing on computed tomography, (5) progressive deterioration of a restrictive impairment of pulmonary function for over 1 year, and (6) respiratory symptoms related to HP for a duration of 6 months or longer.

Antibody measurement and lymphocyte proliferation test (LPT) measurement

IgG and IgA antibodies in sera to avian antigens were detected using enzyme-linked immunosorbent assay with a method as described before.⁶ Peripheral lymphocytes were separated from venous blood and were cultured with pigeon sera, as previously

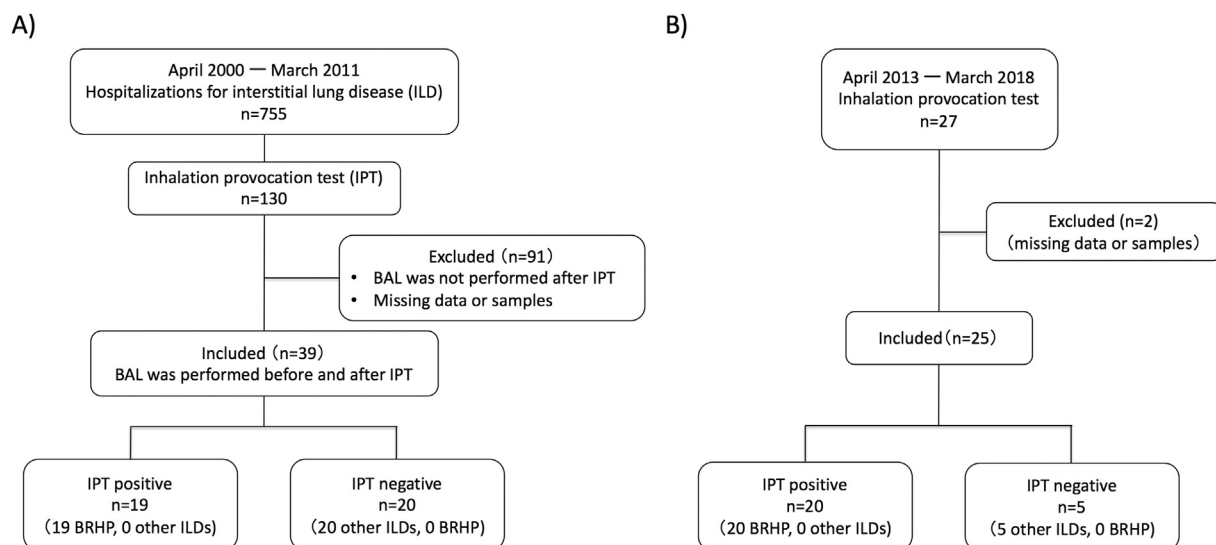


Fig. 1. Diagram illustrating each step in the patient classification process according to the IPT criteria using IPT-PS. (A) patients who underwent BAL before and after the IPT and (B) patients who enrolled for the analysis of serum samples. All of the patients classified as IPT-positive fulfilled the Yoshizawa's criteria for chronic hypersensitivity pneumonitis. IPT-PS, inhalation provocation test prediction score.

described.⁶ The results of LPT were expressed as the stimulation index (SI), which is the geometric mean counts per minute of cultures stimulated with pigeon sera divided by those of unstimulated cultures as a control. The cut off values in specific antibodies and the LPT were determined according to a previous report.¹⁵

Inhalation provocation test

Pigeon dropping extracts (PDE) were prepared and IPT was performed by a previously described method.⁶ Each patient inhaled 2 ml of PDE through a hand nebulizer. The maximal duration of exposure to the antigen was approximately 10 min. White blood cells (WBC), C-reactive protein (CRP), and arterial blood gas were evaluated immediately before the IPT and at 6 and 24 h after the IPT. The result of the IPT was considered positive when the patient scored up to 35 points on the Inhalation Provocation Test-Prediction Score (IPT-PS; $\Delta\text{WBC} [\%] + 2 \times \Delta\text{P} [\text{A-a}] \text{O}_2 [\text{mm Hg}]$), an index confirmed to have both high sensitivity (92.9%) and high specificity (94.7%) in a report from 2015.¹⁸ We adopted the IPT-PS to refine the diagnosis of chronic BRHP in the patients who fulfill the diagnostic criteria of chronic HP.

Bronchoalveolar lavage (BAL)

BAL was performed, as previously described.⁶ BAL was performed using three 50 ml aliquots of sterile 0.9% saline. A baseline BAL was performed at least 2 weeks before the IPT, and a second BAL was performed at 24 h after the IPT. The cellular composition of the BALF was determined by counting 200 cells in a cytospin smear with Wright's staining and May-Grünwald-Giemsa staining.

Multiplex cytokine and chemokine assays

BALF and serum samples were examined with a BioPlex human cytokine Th1/Th2 17-plex panel (Bio-Rad, Richmond, CA, USA) and BioPlex human chemokine assay (Bio-Rad) to measure the concentrations of human granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF- α , CCL2, CCL4, CXCL1, CXCL2, CXCL5, CXCL10, and CCL17. Assays were performed on a Luminex 200 system with xPONENT 3.1 software (Luminex, Austin, TX, USA).

Correlation analysis between cytokine and chemokine changes with blood LPT

The change of each cytokine/chemokine during IPT were shown as delta values (post-IPT minus pre-IPT). Correlation analysis between the delta values of cytokine/chemokine in BALF and serum and the SI values of antigen-induced LPT was performed.

Statistical analysis

All statistical analysis was performed with GraphPad prism software (version 7: GraphPad Software, San Diego, CA, USA). Data are presented as medians with interquartile ranges or as means $\pm$ SEM. Comparisons between-groups were analyzed by the Mann-Whitney U-test or Fisher's exact test, and within-group differences were analyzed by the Friedman test or Wilcoxon signed rank test, as described previously.²⁰ Simple linear regression analyses were used to determine correlations between variables and calculate the Spearman correlation coefficients (r). A p-value of less than 0.05 was considered to be statistically significant.

Results

Patient characteristics

The patient characteristics and clinical data are shown in Table 1. Nineteen of the 39 patients who underwent BAL before and after the IPT, were classified as positive in the IPT, fulfilled the diagnostic criteria for HP, and were diagnosed as chronic BRHP (Fig. 1A). The other 20 of the 39 patients were classified as negative for IPT and diagnosed as other ILDs (14 cases with chronic HP induced by other antigen(s), 4 with ILDs related to collagen vascular diseases, and 2 with IPF). The criteria for positive IPT findings were fulfilled in all of the patients with BRHP but none of the patients with other ILDs. Serum CRP from other ILDs was significantly higher than that from BRHP ($p = 0.03$). The

Table 1
Subject profiles.

(A) Characteristics of the patients in the analysis of BALF			
Characteristics	BRHP (n = 19)	Other ILDs (n = 20)	p value
Age, years	64 (58–74)	66 (59–70)	0.87
Gender (Male/Female)	10/9	16/4	0.09
Cases of smokers, % (No. of current; ex; never)	45.0 (0; 11; 8)	60.0 (2; 12; 6)	0.51
Smoking, pack-years	10 (0–44)	33 (0–54)	0.22
Laboratory findings			
Serum WBC, μL^{-1}	6000 (5000–6400)	6300 (5600–7600)	0.09
Serum CRP, $\text{mg} \cdot \text{dL}^{-1}$	0.10 (0.10–0.20)	0.20 (0.10–0.40)	0.03 *
Serum LDH, $\text{IU} \cdot \text{L}^{-1}$	216 (210–284)	214 (187–237)	0.56
Serum KL-6, $\text{U} \cdot \text{mL}^{-1}$	1070 (874–1620)	1150 (483–1420)	0.74
Serum SP-D, $\text{ng} \cdot \text{mL}^{-1}$	228 (123–284)	193 (160–217)	0.58
Specific antibodies against PDE, %	42.1	70.0	0.11
Lymphocyte proliferation test to avian antigens, % (positive/negative)	68.4 (13/6)	15.0 (3/17)	0.001**
Pulmonary function test			
FVC, % predicted	93.1 (65.0–101)	85.0 (63.6–92.3)	0.31
FEV _{1.0} , % predicted	81.9 (78.6–85.7)	82.7 (63.6–89.7)	0.64
DLco, % predicted	61.9 (38.7–72.4)	62.3 (49.1–74.0)	0.59
(B) Characteristics of the patients in the analysis of serum			
Characteristics	BRHP (n = 20)	Other ILDs (n = 5)	P value
Age, years	59 (63–71)	58 (61–76)	0.43
Gender (Male/Female)	12/8	3/2	1.00
Cases of smokers, % (No. of current; ex; never)	45.0 (2; 7; 11)	60.0 (0; 3; 2)	0.64
Smoking, pack-years	0 (0–38)	11 (0–26.5)	0.86
Laboratory findings			
Serum WBC, μL^{-1}	6600 (4925–7475)	5400 (4650–7650)	0.70
Serum Neutrophil, %	57.9 (53.5–62.6)	57.5 (47.3–65.5)	0.68
Serum CRP, $\text{mg} \cdot \text{dL}^{-1}$	0.11 (0.07–0.20)	0.07 (0.02–1.57)	0.27
Serum LDH, $\text{IU} \cdot \text{L}^{-1}$	221 (209–238)	219 (203–233)	0.73
Serum KL-6, $\text{U} \cdot \text{mL}^{-1}$	1204 (744–1594)	698 (526–4023)	0.61
Serum SP-D, $\text{ng} \cdot \text{mL}^{-1}$	256 (138–439)	298 (187–1205)	0.12
Specific antibodies against PDE, %	50.0	60.0	>0.99
Lymphocyte proliferation test to avian antigens, % (positive/negative)	77.7 (7/2)	0.0 (0/4)	0.02 *
Pulmonary function test			
FVC, % predicted	74.8 (64.8–90.9)	85.0 (63.6–92.3)	0.39
FEV _{1.0} , % predicted	81.9 (69.8–88.8)	82.7 (63.6–89.7)	1.00
DLco, % predicted	57.8 (35.5–73.0)	63.4 (62.8–85.6)	0.26

Data are presented as the numbers or medians (25th and 75th percentiles). * $p < 0.05$, ** $p < 0.01$.

positive rate of antigen-induced LPT was significantly higher in patients with BRHP than those with other ILDs ($p = 0.001$). No significant differences were observed in the clinical data, other laboratory findings, or pulmonary function test.

Twenty-five consecutive patients were newly enrolled for the analysis of serum samples (Fig. 1B). All 20 of the patients were considered positive for IPT, met the diagnostic criteria for HP, and were diagnosed as chronic BRHP. Five patients tested negative in the IPT and were diagnosed as other ILDs (3 cases with chronic HP induced by antigen(s) other than avian antigen, 1 case with IPF, and 1 case with pleuroparenchymal fibroelastosis). The positive rate of antigen-induced LPT was significantly higher in patients with BRHP than those with other ILDs ($p = 0.02$). There were no significant differences in patient characteristics, other laboratory findings, or pulmonary function tests between the patients with BRHP and those with other ILDs (Table 1).

BALF cellularity before and after the IPT

Baseline BALs were performed at $5.6 (\pm 2.1)$ weeks and $4.9 (\pm 1.9)$ weeks before the IPT in 19 BRHP patients and 20 other ILD patients. Figure 2 and Supplementary Table 1 shows the BALF findings before and after the IPT. No significant differences were found in the baseline characteristics of the BALF between the BRHP patients and other ILD patients. The total number of BALF cells from the BRHP patients was significantly elevated after the IPT ($p = 0.001$) (Fig. 2A). The BRHP group showed a decrease in the proportion of BALF macrophages ($p = 0.01$) and increase in the proportion of BALF neutrophils ($p < 0.001$), after the IPT (Fig. 2B, C). No significant difference was found in the proportion of BALF lymphocytes between the two groups (Fig. 2D). On the other hand, a strong correlation was found between the difference of neutrophils during the IPT and the IPT-PS ($r = 0.71$, $p < 0.001$).

Measurement of cytokines and chemokines in BALF

Figure 3, 4 show the cytokine and chemokine profiles in the BALF before and after the IPT. There were no significant differences in the baseline BALF profiles (cellularity, cytokines and chemokines) between BRHP and other ILDs (Supplementary Table 2). The BALF neutrophil-chemoattractant cytokines after the IPT were increased in the BRHP patients (Fig. 3). The BALF G-CSF, IL-6, and IL-17 levels were significantly elevated after the IPT only in the BRHP patients ($p < 0.001$, $p < 0.001$, and $p = 0.02$, respectively) (Fig. 3A, B, D). Increases in the neutrophil chemoattractant chemokines IL-8 ($p = 0.006$) (Fig. 3C) and CXCL2 ($p = 0.009$) were also observed after the IPT in the BRHP patients. No increases of IL-1 β , CXCL1, or CXCL5 were observed in either the BRHP patients ($p = 0.92$, $p = 0.86$, and $p = 0.46$, respectively) or other ILD patients ($p = 0.26$, $p = 0.95$, and $p = 0.66$, respectively) (Supplementary Fig. 1). CXCL10 (Th1 chemokine) was significantly elevated after the IPT in the BRHP patients ($p = 0.02$), but no significant change in CCL17 (Th2 chemokine) was found in either the BRHP patients or other ILD patients ($p = 0.42$, and $p = 0.06$, respectively) (Fig. 4A, B). Both IL-12 (Th1 cytokine) and IL-13 (Th2 cytokine) were not detectable. A statistically significant increase of CCL2 was also observed in the BRHP patients ($p = 0.01$) (Fig. 4C). None of the other cytokines were detectable in the BALF from either the BRHP patients or other ILD patients.

Relationship between the BALF neutrophils and cytokine and chemokine levels

Significant positive correlations were found between the differences in the BALF neutrophil percentages and differences in the levels of BALF G-CSF, IL-6, IL-8, and IL-17 before and after the IPT ($r = 0.55$; $p < 0.001$, $r = 0.61$; $p < 0.001$, $r = 0.53$; $p < 0.001$, and $r = 0.53$; $p < 0.001$, respectively) (Supplementary Fig. 2). The differences in the BALF neutrophil percentages were also significantly

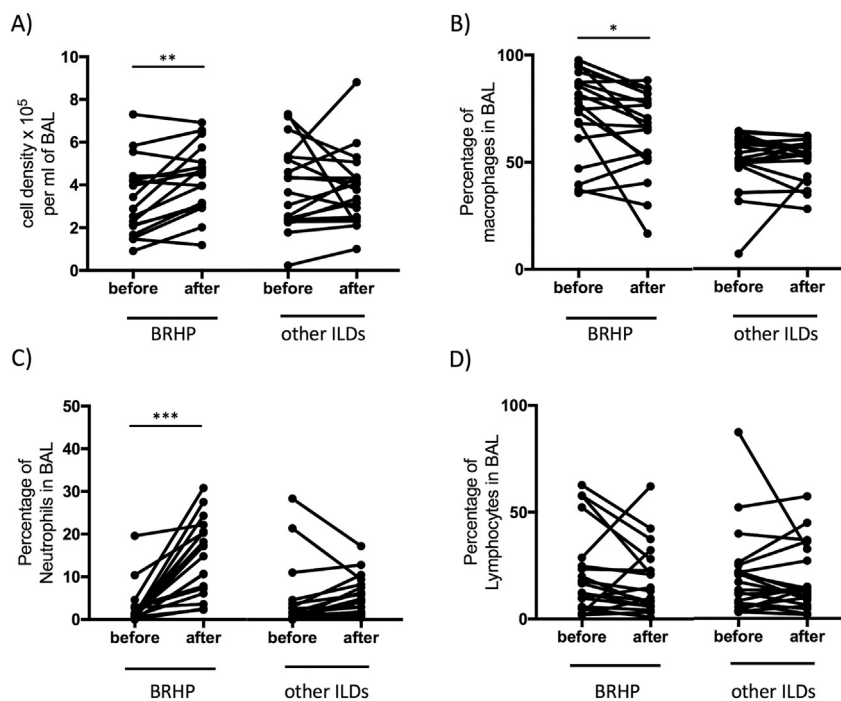


Fig. 2. Changes in BALF cell counts between before and 24 h after the IPT. (A) cell density (compared between before and after the IPT; BRHP, $p = 0.002$; other ILDs, $p = 0.63$), (B) macrophages ($p = 0.02$, $p = 0.82$), (C) neutrophils ($p < 0.001$, $p = 0.31$), (D) lymphocytes ($p = 0.13$, $p = 0.85$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

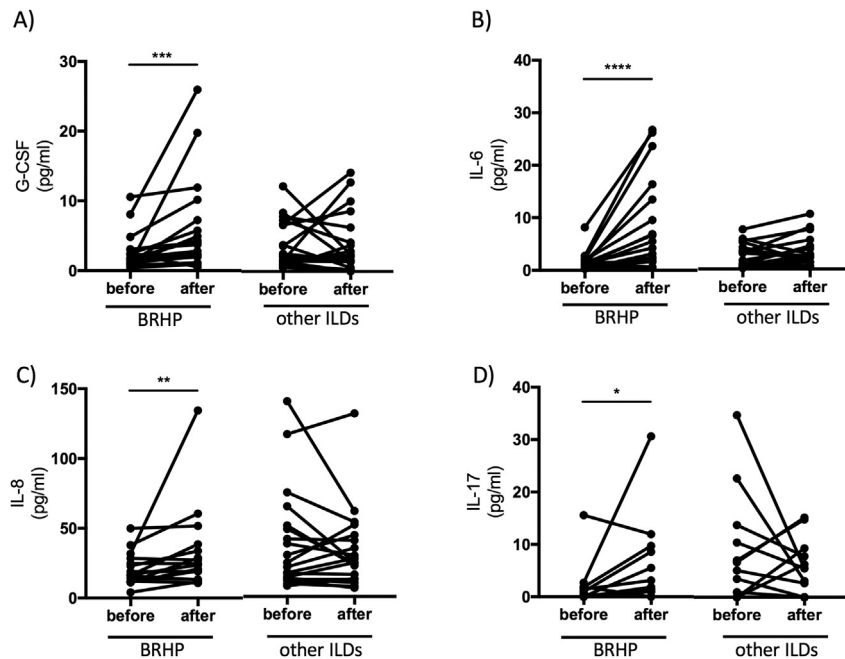


Fig. 3. Changes of neutrophil-chemoattractant cytokines and chemokines in BALF between before and 24 h after the IPT. (A) G-CSF (comparison between before and after the IPT; BRHP, $p < 0.001$; other ILDs, $p = 0.93$), (B) IL-6 ($p < 0.001$, $p = 0.28$), (C) IL-8 ($p = 0.006$, $p = 0.51$), (D) IL-17 ($p = 0.02$, $p = 0.83$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

correlated with the difference in the level of BALF CXCL2 after the IPT ($r = 0.65$; $p < 0.001$), whereas no significant correlation was found in the level of BALF CXCL1 ($r = 0.01$; $p = 0.95$).

Measurement of cytokine and chemokine levels in serum

The white blood cell count and neutrophil count in peripheral blood were significantly elevated at 6 h after the IPT only in the BRHP patients ($p < 0.001$ and $p < 0.001$, respectively). Figure 5, 6 show the cytokine and chemokine profiles in serum before the IPT and at 6 and 24 h after the IPT. The levels of G-CSF, IL-6, IL-8, CXCL1, and CXCL2 in serum were elevated at 6 h after the IPT ($p = 0.005$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively) (Fig. 5A-E) and subsequently reverted to their baseline levels at 24 h after the IPT. No significant elevation of the neutrophil chemoattractant CXCL5 was observed in either patient group during the IPT ($p = 0.33$ and $p = 0.90$) (Fig. 5F). Serum levels of the Th1 cytokines/chemokines IFN- γ and CXCL10 were elevated at 6 h after the IPT in the BRHP patients ($p < 0.001$ and $p = 0.03$), while the levels of Th2 cytokine/chemokines IL-5 and CCL17 showed no significant change ($p = 0.70$ and $p = 0.38$) (Fig. 6A-D). The BRHP patients also exhibited significant elevations of CCL2 and CCL4 at 6 h

after the IPT ($p < 0.001$ and $p = 0.02$) (Fig. 6E, F). Further, significant increases in the inflammatory cytokine TNF- α and anti-inflammatory cytokine IL-10 were observed at 6 h after the IPT in the BRHP patients ($p = 0.01$ and $p = 0.02$) (Fig. 6G, H). None of the other serum cytokines, namely, GM-CSF, IL-1 β , IL-2, IL-4, IL-7, IL-12, IL-13 and IL-17, were detected in the measurements at any time point.

Correlation analysis between cytokine and chemokine changes with blood LPT

The positive rate of antigen-induced blood LPT was significantly higher in patients with BRHP than those with other ILDs, but the positive rate of antibodies against avian antigen did not differ in both groups (Table 1). Although there was no significant difference in the changes of all cytokines and chemokines during IPT with or without positive titer for antibodies against avian antigen, significant differences were observed in serum G-CSF, IL-6, IFN- γ , CXCL10, TNF- α and BALF IL-17 concentrations between positive and negative LPT. Table 2 shows correlation results of cytokine and chemokine changes in IPT with the SI values of blood LPT. The delta values in IPT were correlated with

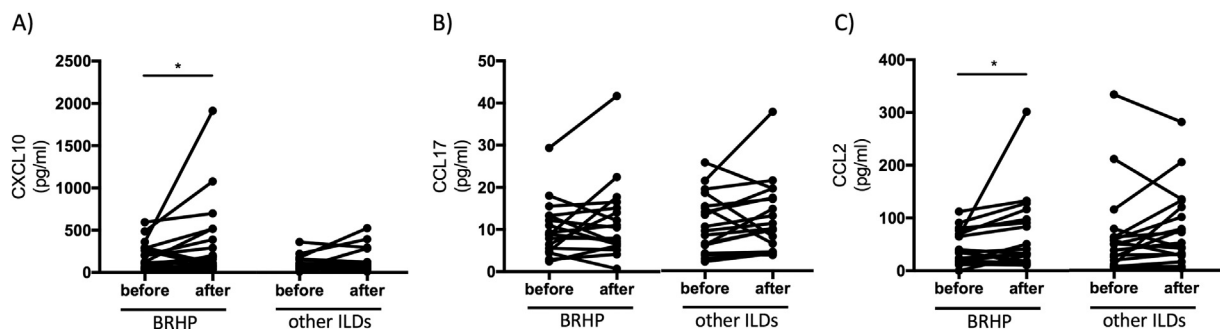


Fig. 4. Changes in BALF cytokines and chemokines for the chemotaxis of Th1/Th2 and BALF monocytes/macrophages between before and 24 h after the IPT. (A) CXCL10 (comparison between before and after the IPT; BRHP, $p = 0.02$; other ILDs, $p = 0.65$), (B) CCL17 ($p = 0.42$, $p = 0.06$), and (C) CCL2 ($p = 0.02$, $p = 0.26$). * $p < 0.05$.

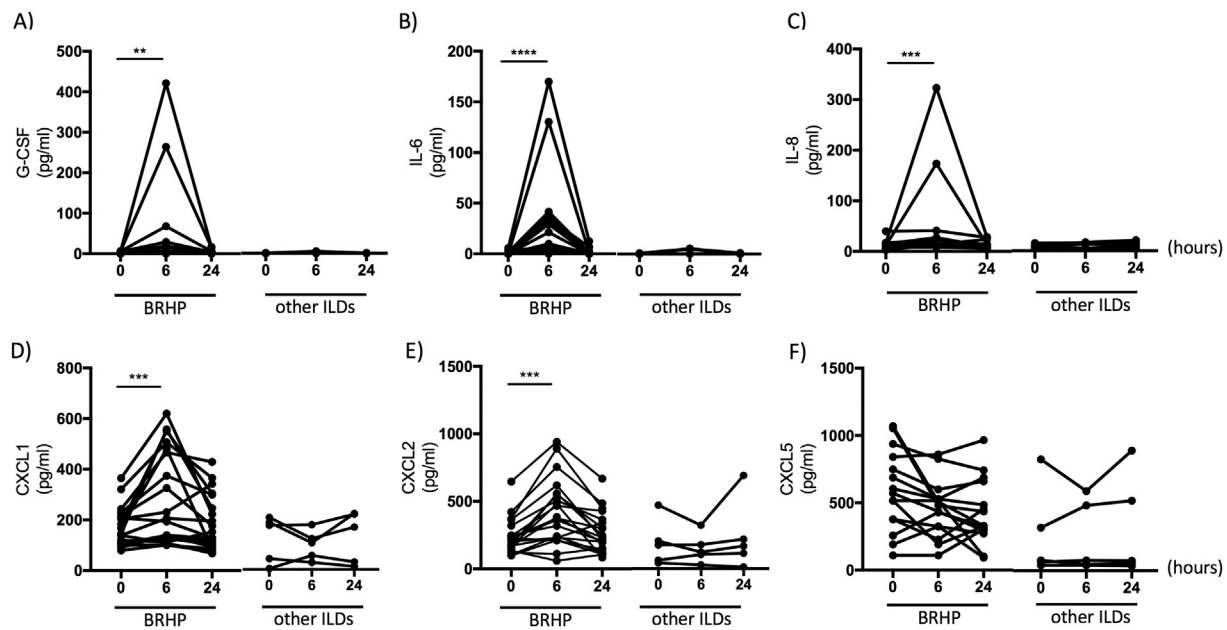


Fig. 5. Changes in neutrophil-chemoattractant cytokines and chemokines in serum between before the IPT and 6 h and 24 h after the IPT. (A) G-CSF (Friedman test; BRHP, $p = 0.005$; other ILDs, $p = 0.15$), (B) IL-6 ($p < 0.001$, $p = 0.15$), (C) IL-8 ($p < 0.001$, $p = 0.52$), (D) CXCL1 ($p < 0.001$; $p = 0.95$), (E) CXCL2 ($p < 0.001$, $p = 0.52$), and (F) CXCL5 ($p = 0.33$, $p = 0.90$). ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

the SI values of blood LPT in serum G-CSF, IL-6, IFN- γ , CXCL10, TNF- α and BALF IL-17.

Discussion

This study is the first to demonstrate how the immunological reaction occurs just after the inhalation of causative antigens in the chronic phase of HP. The results showed that neutrophils and the cytokine/chemokine-associated neutrophil and Th1 responses were increased in both the serum and BALF obtained from patients with chronic BRHP in the early phase after exposure to the offending antigen.

First, we began our study by evaluating a number of neutrophil-chemoattractant cytokines and chemokines, namely, G-CSF, IL-6, IL-8, IL-17, IL-1 β , CXCL1, CXCL2, and CXCL5. Marked neutrophilic inflammation can be observed in the lungs of HP patients in the acute phase just after an inhalation of causative antigens.^{4–7} Our experiments demonstrated elevated levels of IL-8 after the IPT in both serum and BALF from chronic HP patients, and the change of BALF IL-8 was positively correlated with the difference in the percentages of BALF neutrophils during the IPT. IL-8 exerts a potent chemotactic effect and activates neutrophils.²² Increases of BALF IL-8 and alveolar macrophages obtained from HP patients demonstrate the crucial role IL-8 plays in inflammation and disease

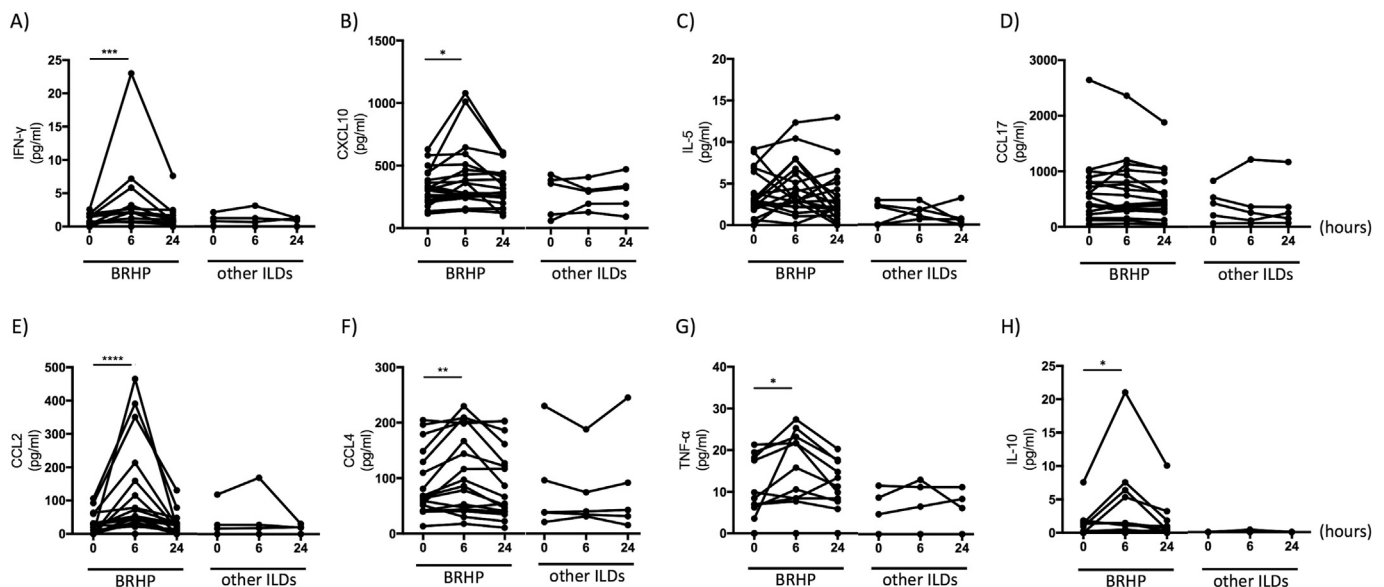


Fig. 6. Changes in serum cytokines and chemokines for the chemotaxis of T-helper type 1/2 (Th1/Th2) and monocytes/macrophages between before the IPT and 6 h and 24 h after the IPT. (A) IFN- γ (Friedman test; BRHP, $p < 0.001$; other ILDs, $p = 0.88$), (B) CXCL10 ($p = 0.03$, $p = 0.95$), (C) IL-5 ($p = 0.70$, $p = 0.80$), (D) CCL17 ($p = 0.38$, $p > 0.9999$), (E) CCL2 ($p < 0.001$, $p = 0.26$), (F) CCL4 ($p = 0.02$, $p = 0.95$), (G) TNF- α ($p = 0.02$, $p = 0.94$), and (H) IL-10 ($p = 0.02$, $p = 0.44$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 2

Correlation analysis between the changes in serum and BALF cytokine/chemokine values with the stimulation index values of blood lymphocyte proliferation test.

Correlations with SI values of LPT	serum		BALF	
	(n = 13)		(n = 39)	
	r value	p value	r value	p value
Δ G-CSF	0.60	0.04	0.22	0.18
Δ IL-6	0.64	0.02	0.14	0.40
Δ IL-8	0.15	0.63	0.13	0.43
Δ IL-17	NA	NA	0.45	0.005
Δ CXCL1	0.28	0.38	0.02	0.89
Δ CXCL2	0.34	0.27	0.19	0.26
Δ CXCL5	0.02	0.95	0.04	0.78
Δ IFN-γ	0.63	0.04	NA	NA
Δ CXCL10	0.59	0.04	0.10	0.56
Δ IL-5	0.25	0.43	−0.11	0.49
Δ CCL17	0.07	0.83	−0.11	0.49
Δ CCL2	0.41	0.18	−0.19	0.24
Δ CCL4	0.32	0.31	−0.05	0.77
Δ TNF-α	0.88	0.003	NA	NA
Δ IL-10	0.57	0.15	NA	NA

LPT, lymphocyte proliferation test; SI, stimulation index.

progression in HP.^{8,22–24} Denis *et al.* also reported a decrease in the secretion of IL-8 by alveolar macrophages after treatment in BALF from patients with farmer's lung.²³ Moreover, neutrophilic infiltration via the IL-17A-CXCL5 pathway played important roles in both a murine model of acute BRHP²⁵ and a *Saccharopolyspora rectivirgula* (SR) murine model of HP, in previous experiments by our group.^{26,27} IL-17A, G-CSF, IL-6, CXCL1, CXCL2, CXCL5 and TNF-α were elevated together with neutrophil infiltration in the acute phase in antigen-challenged mice used for these studies. IL-17 was also shown to aggregate neutrophils in the lung with the production of IL-6, IL-8, CXCL2, TNF-α, and IL-1β.²⁸ Similarly, a comparison of gene expression between patients with HP and patients with IPF revealed an upregulation of the IL-17 gene in HP.²⁹ In the present study we observed an increase of IL-17 after the IPT in BALF from patients with BRHP, not but in serum. Although all of the patients who underwent BAL had no detectable radiologic changes with chest radiograph during IPT, the percentage of neutrophil was elevated at 24 h after IPT. Therefore, we suggested that the change of BALF IL-17 reflected local inflammation in lung.

Second, acute HP is considered to be a Th1-mediated disease dependent on Th1 cytokines and chemokines such as IFN-γ and CXCL10. CXCL10 plays a role in the induction of the Th1 response and activation of antigen-specific effector T cells in an SR murine model of HP, with the production of TNF-α and CCL4.³⁰ In earlier experiments by our group, however, we found that the Th1/Th2 balance shifted to a Th2 predominance in both chronic HP patients and a PDE murine model of chronic HP with fibrosis.^{10,31} Although our study focused in patients with chronic BRHP, the patients exhibited increases in the Th1 cytokine and chemokine (IFN-γ and CXCL10) with no accompanying changes in the Th2 cytokines or chemokine (IL-4, IL-5, and CCL17) after the IPT. CCL4, on the other hand, was increased after the IPT in the serum of BRHP patients but not in the BALF. CCL4 is produced by various cells such as macrophages, neutrophils, and T- and B-lymphocytes and plays a crucial role in T-cell chemotaxis (especially Th1 inflammation).³² SR stimulated the expression of CCL4 in both an in vitro experiment with murine alveolar macrophage cell lines (MH-S) and an in vivo experiment with an SR murine model of HP.^{27,30} Hence, CCL4 is also likely to be associated with the initiation of inflammation in HP. If such an association can be confirmed, we can infer that a Th1 immunological reaction akin to acute HP is induced in the acute phase after exposure to specific antigens in chronic BRHP.

Third, the important anti-inflammatory cytokine IL-10 was elevated after the IPT in BALF from BRHP patients. In earlier studies we found that IL-10 may play a protective role in isocyanate-induced HP.³³ The analysis of gene expression in HP also showed that the IL-10 was more upregulated than IPF.²⁹ Further, an impairment of T-regulatory cells due to increased IL-17 production has been demonstrated in HP.³⁴ Though IL-10 increased after the IPT in BRHP serum, the amount of IL-10 produced may have been relatively insufficient to suppress the exacerbated immune response of BRHP.

Fourth, CCL2 is produced by a variety of cells such as alveolar epithelial cells, monocytes, macrophages, and endothelial cells, and regulates the migration and infiltration of monocytes and the recruitment of T cells.^{35,36} CCL2 has been reported to increase and to correlate significantly with the number of lymphocytes in BALF from HP patients.⁸ The production of CCL2 is also induced in BALF from the SR murine model of HP.³⁷ Judging from the significant increases observed in CCL2 in the serum and BALF from BRHP patients after the IPT in our study, we surmised that CCL2 contributes to inflammatory responses involving macrophages, monocytes, and T cells during exposure to specific antigens in HP.

Antigen-experienced memory T cells induce Th1/Th2 immunologic responses in HP.³⁸ Several studies demonstrated that a skewing toward Th2 responses as opposed to Th1 responses was observed in chronic HP, as mentioned above.^{10,31,38} Barrera *et al.* revealed that the T cell exhaustion is attributed to skewing toward Th2 activity in chronic HP.³⁸ On the other hand, we previously reported that avian antigens exist in the household environments of BRHP patients in spite of antigen avoidance and the presence of avian antigens can predict the progression of chronic BRHP.^{39,40} Most patients of chronic HP, especially in BRHP, are slowly progressing with no history of acute episode because of a unrecognized exposure to low-dose avian antigens and developing pulmonary fibrosis.⁴¹ Therefore, it is possible that exposure to the causative antigen induces the activation of memory T cells and repeated Th1-dominant immune responses in chronic HP with insidious progression.

While our study is the first to evaluate the action of cytokines and chemokines during the IPT in the serum and BALF from patients with HP, we acknowledge that it has several limitations. First, we enrolled a relatively small number of patients with ILDs, and the numbers of patients from which serum and BALF were obtained differed. Second, this study enrolled only patients with BRHP and shed no light on HP caused by other antigens. The difference in inflammation profiles between BRHP and HP induced by fungi has been reported previously.²⁰ Although chronic HP caused by other antigens certainly requires study, BRHP is the most common variant of chronic HP in Japan,¹³ and meaningful insight can be drawn from the analysis of inflammatory and immunological responses of the chronic form of the disease. Third, the BAL was performed only before and at 24 h after the IPT. As described before, most inflammatory mediators in serum were elevated at 6 h and almost fully returned to the baseline levels at 24 h. Hence, the variation in cytokine/chemokine levels may have been underestimated when the levels were only determined before and at 24 h after IPT, even though the neutrophils increased at 24 h after the IPT in BALF from the patients with BRHP. Fourth, the correlations between the changes of cytokine/chemokine in IPT and the SI values of LPT should be carefully interpreted because of risks in multiple comparisons. Further study must be conducted to estimate the correlations.

In conclusion, this study demonstrated that neutrophilic and Th1-mediated inflammation occurs in patients with chronic BRHP within 6 h following inhalation of an offending antigen, as evidenced by increases in cytokine-/chemokine-associated neutrophil and Th1 responses. Our findings indicate that exposure to the causative antigen provokes an inflammatory and immunological response similar to acute HP even in the chronic phase of HP.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2018.12.010>.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

YM had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. YM and YI contributed to the conception and design of the study; YM, YI, MI, HF, TH, TK and TT contributed to the analysis and interpretation of the data; YM, MI, HF and YI contributed to the drafting of the submitted article; YM and HF contributed to critical revision of the manuscript for important intellectual property.

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Supplementary Table 1. The cellular bronchoalveolar lavage fluid profile of pre and post inhalation provocation test.

	BRHP (n = 19)		Other ILDs (n = 20)	
	Pre-IPT	Post-IPT	Pre-IPT	Post-IPT
Cell density, $\times 10^5 \cdot \text{ml}^{-1}$	3.44 (2.09-4.70)	3.97 (2.93-5.06)	3.36 (2.32-5.29)	3.57 (2.46-4.35)
Macrophages, %	77.5 (61.1-87.3)	66.5 (50.8-79.2)	76.6 (71.5-89.9)	80.4 (62.0-87.6)
Lymphocytes, %	16.6 (5.7-28.7)	13.0 (5.9-28.1)	15.3 (7.18-24.2)	13.1 (6.88-31.1)
Neutrophils, %	1.1 (0.5-2.8)	17.2 (7.3-20.6)	1.05 (0-3.12)	3.8 (1.25-7.15)
Eosinophils, %	1.0 (0.6-2.0)	1.0 (0-4.1)	0.55 (0-1.53)	0.95 (0.1-1.73)
CD4/CD8 ratio	2.05 (0.97-5.99)	1.50 (1.06-3.38)	2.57 (1.60-4.87)	1.58 (0.96-2.50)

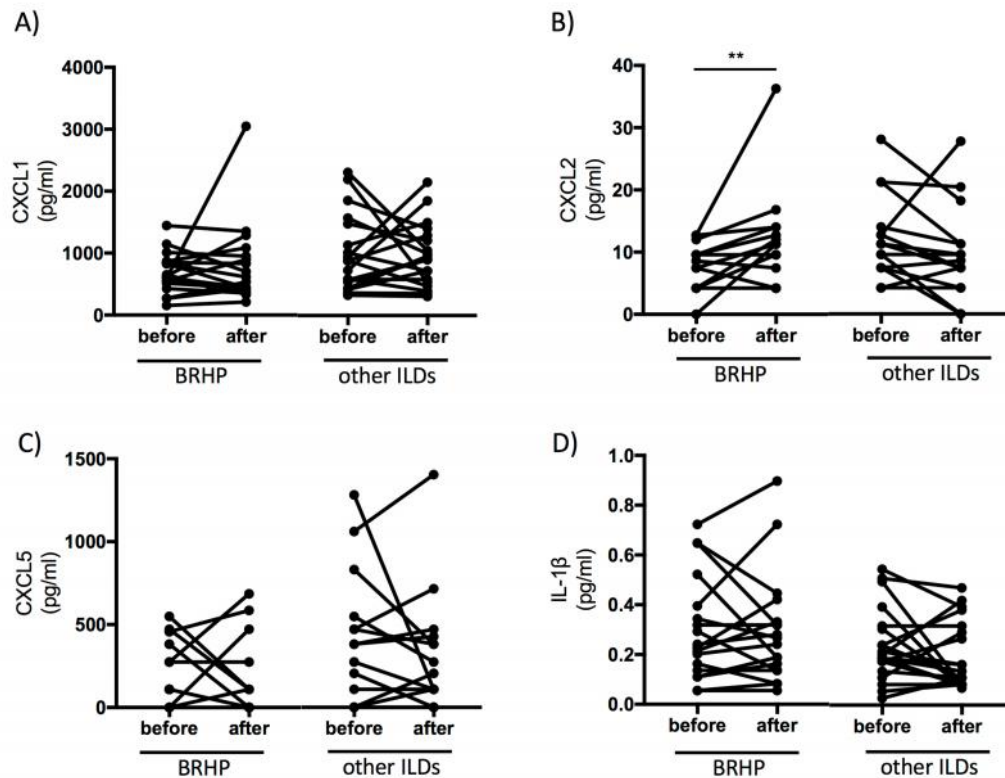
Data are presented as median (25th and 75th percentiles).

Supplementary Table 2. Comparison of baseline BALF in cellularity, cytokines and chemokine profiles.

	BRHP (n = 19)	Other ILDs (n = 20)	P value
Cellular profile			
Cell density, $\times 10^5 \cdot \text{ml}^{-1}$	3.44 (2.09-4.70)	3.36 (2.32-5.29)	0.5687
Macrophages, %	77.5 (61.1-87.3)	76.6 (71.5-89.9)	0.6122
Lymphocytes, %	16.6 (5.7-28.7)	15.3 (7.18-24.2)	0.9502
Neutrophils, %	1.1 (0.5-2.8)	1.05 (0-3.12)	0.8066
Eosinophils, %	1.0 (0.6-2.0)	0.55 (0-1.53)	0.0611
CD4/CD8 ratio	2.05 (0.97-5.99)	2.57 (1.60-4.87)	0.6955
Cytokine and chemokine			
G-CSF, $\text{pg} \cdot \text{mL}^{-1}$	1.78 (1.07-3.04)	2.16 (1.29-6.70)	0.4731
IL-6, $\text{pg} \cdot \text{mL}^{-1}$	1.13 (0.52-1.79)	2.35 (0.54-4.40)	0.2545
IL-8, $\text{pg} \cdot \text{mL}^{-1}$	16.3 (11.8-26.6)	24.3 (13.0-50.9)	0.1157
IL-17, $\text{pg} \cdot \text{mL}^{-1}$	1.20 (0-2.06)	3.51 (0-10.5)	0.4929
CXCL10, $\text{pg} \cdot \text{mL}^{-1}$	123.8 (59.4-288.4)	81.9 (46.8-151.3)	0.0919
CCL17, $\text{pg} \cdot \text{mL}^{-1}$	8.84 (5.16-13.2)	9.49 (4.09-15.3)	0.8204
CCL2, $\text{pg} \cdot \text{mL}^{-1}$	36.0 (17.7-71.1)	49.9 (17.7-67.4)	0.8084
CXCL1, $\text{pg} \cdot \text{mL}^{-1}$	619 (493-857)	880 (543-1479)	0.1687
CXCL2, $\text{pg} \cdot \text{mL}^{-1}$	7.46 (4.20-9.58)	9.58 (6.65-12.7)	0.0767
CXCL5, $\text{pg} \cdot \text{mL}^{-1}$	110 (0-382)	239 (0-471)	0.5559
IL-1 β , $\text{pg} \cdot \text{mL}^{-1}$	0.24 (0.13-0.43)	0.20 (0.15-0.32)	0.4295

Data are presented as median (25th and 75th percentiles).

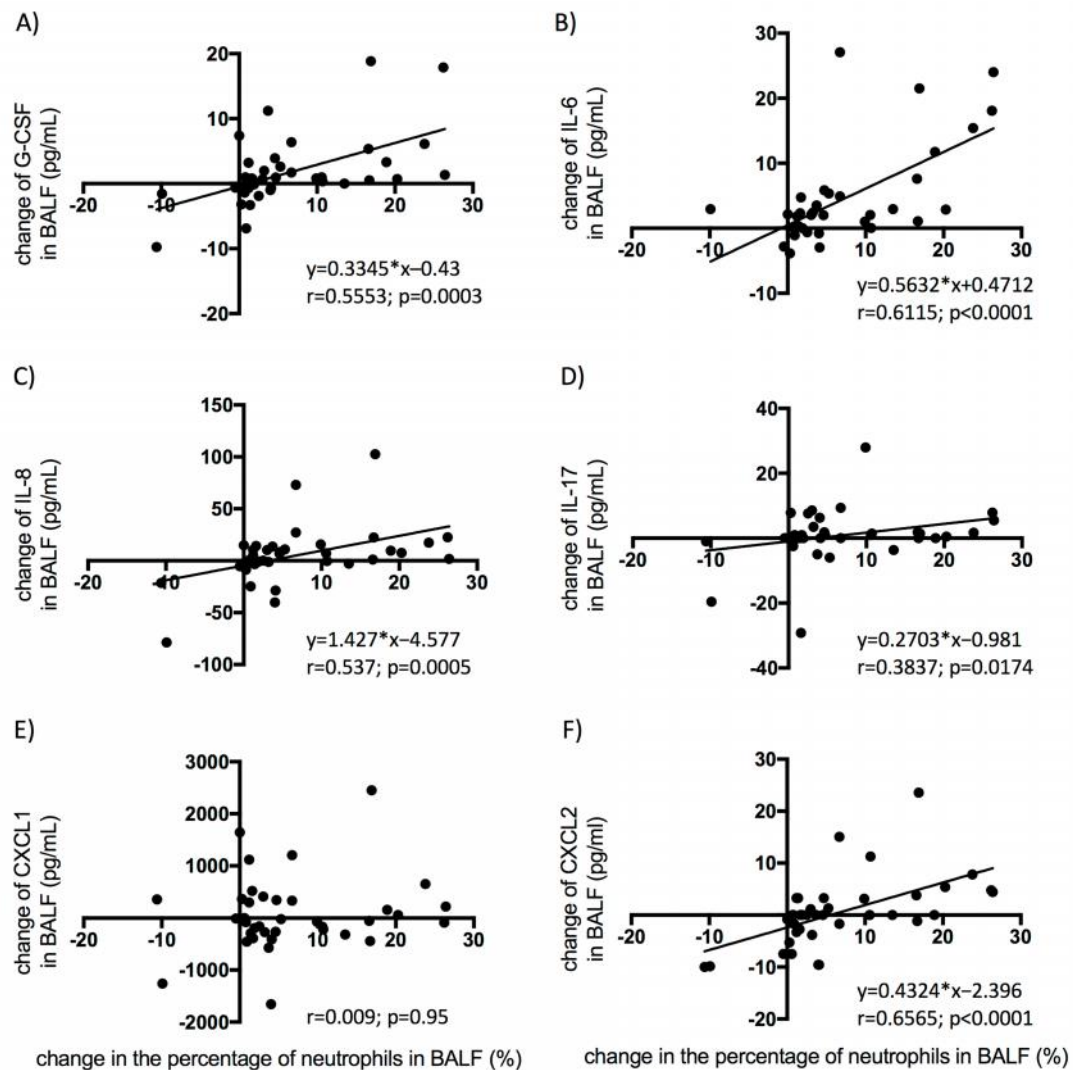
Supplementary Figure 1.



Supplementary Figure 1.

Changes in neutrophil-chemoattractant cytokines and chemokines in BALF between before the IPT and 24h after the IPT. (A) CXCL1 (comparison between before and after the IPT; BRHP, $p=0.8650$; other ILDs, $p=0.9530$), (B) CXCL2 ($p=0.0098$, $p=0.0633$), (C) CXCL5 ($p=0.4604$, $p=0.6680$), (D) IL-1 β ($p=0.9231$, $p=0.2598$).

Supplementary Figure 2.



Supplementary Figure 2.

Correlations between the differences in the BALF neutrophil percentages and differences in the cytokine/chemokine levels between before and 24 hours after the IPT in all subjects. The abscissas show the differences in the BALF neutrophil percentages during the IPT and the ordinates represent the changes in the levels of (A) G-CSF, (B) IL-6, (C) IL-8, (D) IL-17, (E) CXCL1 and (F) CXCL2, respectively, during the IPT.