either of the STAT1-EGFP mutants of K278E, G384D, and T385M was transiently transfected into HeLa cells (Fig. 2D). Moreover, impaired dephosphorylation was observed in the endogenous WT STAT1 when either of the STAT1-EGFP mutants was transiently expressed in HeLa cells (Fig. 3). Although these mechanisms were not fully elucidated in our study, they suggest that heterodimers of WT and the mutants of STAT1 could cause impaired dephosphorylation, as do homodimers of the STAT1 GOF mutants.

Th17 population was reduced in patients 1 and 3 but not in patient 2

Impaired differentiation of Th17 cells was indicated to be associated with the development of CMCD in patients with *STAT1* GOF mutations (3, 4, 13, 14). Therefore, we studied the proportion of $CD4^+IL\text{-}17A^+$ cells among $CD4^+$ cells in the patients with novel *STAT1* GOF mutations following PMA plus IOM stimulation for 6 h (Fig. 4). Patient 1 with a heterozygous K278E mutation and patient 3 with a heterozygous G384D mutation were shown to have relatively reduced $CD4^+IL\text{-}17A^+$ cells (0.28 and 0.24% of $CD4^+$ cells, respectively). In contrast, patient 2 with the same heterozygous G384D mutation as patient 3 had almost normal $CD4^+IL\text{-}17A^+$ cells (0.58% of $CD4^+$ cells). These findings prompted us to further study Th17-associated cytokines IL-17A, IL-17F, and IL-22 following various stimulations.

Evaluation of the profile of Th17-associated cytokine production

First, cytokine production was analyzed in the supernatant of the purified $CD4^+$ cells following PMA plus IOM stimulation for 6 h, the same stimulation as performed in evaluating Th17 population (Fig. 5). The purity of $CD4^+$ cells positively selected by CD4 microbeads was 97.5–99.7%. Production of the Th17-associated cytokines IL-17A, IL-17F, and IL-22 was not significantly reduced in the patients with *STAT1* GOF mutations compared with healthy controls. We then studied cytokine production after more physiologically relevant stimulations, that is, *Candida* or CD3/28 stimulations. Production of all the Th17-associated cytokines in PBMCs and $CD4^+$ cells was significantly reduced in response to *Candida* stimulation. Alternatively, although production of IL-17A and IL-22 was significantly reduced, IL-17F production was comparable to healthy controls following CD3/28 stimulation. Additionally, IFN-γ and IL-4, the principal cytokines of Th1 and Th2, respectively, were analyzed (Fig. 5). IFN-γ and IL-4 production from patients' PBMCs or $CD4^+$ cells was not significantly different from that of controls following *Candida* or CD3/28 stimulation. Each sample without stimulation showed nil or negligible cytokine production (data not shown).

Anti–IL-17F autoantibody was present in sera from CMC patients with STAT1 GOF

In 2010, two reports indicated the neutralizing Abs against IL-17A, IL-17F, and IL-22 would be the etiology of CMC in APS1 patients (16, 17). It is possible that CMCD in patients with *STAT1* GOF mutations could be also attributable to neutralizing Abs based on the fact that these patients often manifest autoimmune diseases. Thus, we analyzed autoantibodies against various cytokines, including Th17-associated cytokines, first with HRP-conjugated goat anti-human IgG Ab. Immunoblot analysis of sera from two APS1 patients showed various autoantibodies against Th17-associated cytokines in addition to IFN-α as reported previously (Fig. 6) (16, 17). We then studied sera from 17 STAT1 GOF patients and demonstrated the exclusive presence of anti–IL-17F IgG autoantibody (thereafter described as anti–IL-17F autoantibody) in 11 patients (64.7%) (Fig. 6). Two patients with STAT3-deficient HIES also showed anti–IL-17F autoantibody (Fig. 6). Immunoblot analysis of serially diluted sera from STAT1 GOF patients demonstrated that the titer of this autoantibody ranged from 1:2,560 to 1:20,480, and 1:2,560 was the most frequently observed (data not shown). Furthermore, with HRP-conjugated goat anti-human IgA Ab, anti–IL-17F IgA autoantibody was demonstrated in sera from six patients (P1, P6, P8–P11) who were all positive for anti–IL-17F autoantibody (data not shown). In contrast, none of the present patients was demonstrated to have autoantibodies against IL-1β, IL-6, and TGF-β1 that could be associated with Th17 differentiation (data not shown). Each of the anti–IL-17F, anti–IFN-α, and anti–IL-6 autoantibodies was detected in 1 among 21 healthy controls without any overlaps (Fig. 6 and data not shown).

We confirmed the results by more than two independent experiments.

Neutralizing activity was not demonstrated when IL-17F–induced IL-6 production was studied in healthy control fibroblasts in

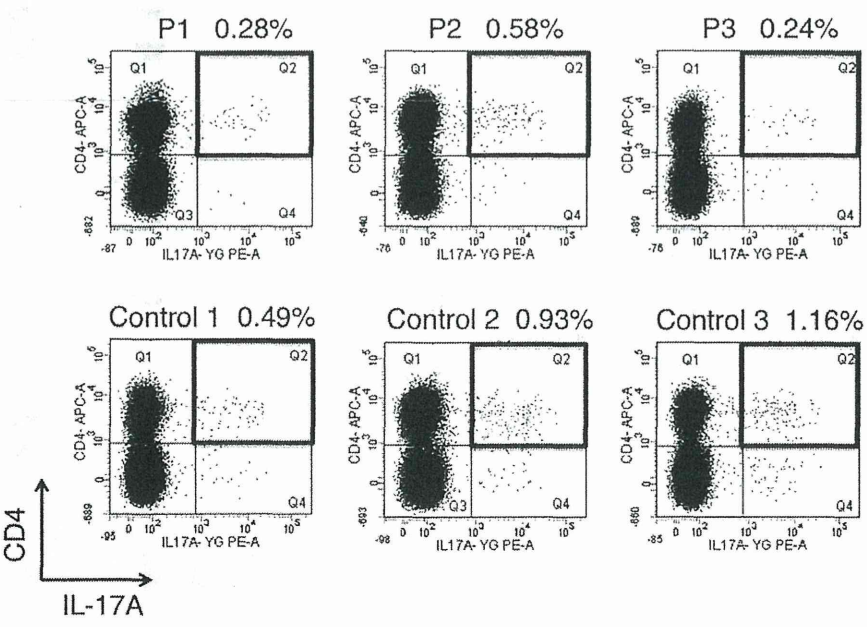

FIGURE 4. Patients 1 and 3 had reduced $CD4^+IL17A^+$ cells in response to PMA plus IOM stimulation, whereas patient 2 had a normal proportion of $CD4^+IL\text{-}17A^+$ cells compared with controls.

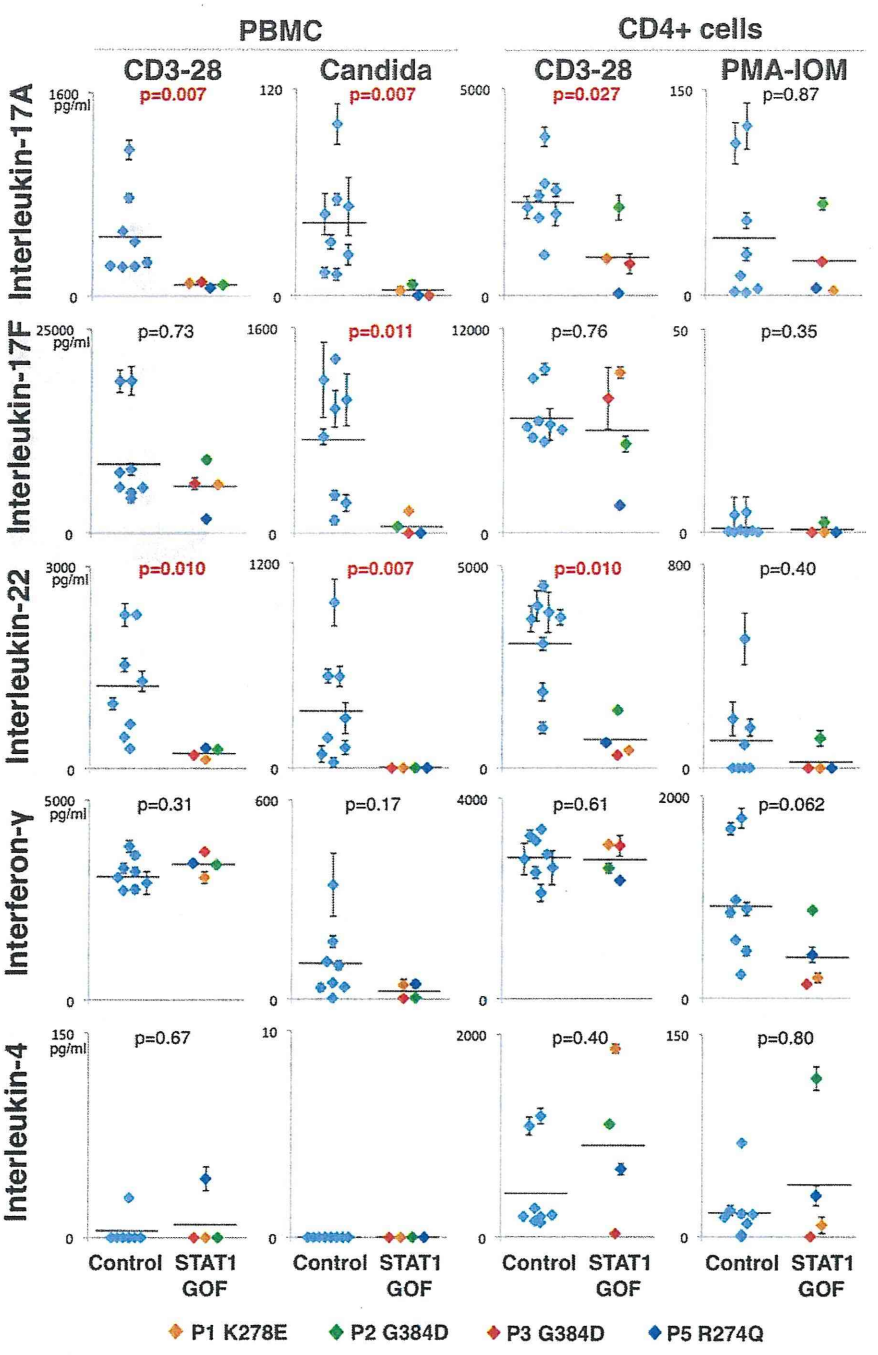

FIGURE 5. IL-17A and IL-22 production from PBMCs and CD4$^+$ cells was significantly reduced in four patients with STAT1 GOF in response to CD3/28 or *Candida* stimulations, whereas IL-17F production was comparable to healthy controls in response to CD3/28 stimulation. Productions of IL-17A, IL-17F, IL-22, IFN-γ, and IL-4 from PBMCs and CD4$^+$ cells of the patients with STAT1 GOF mutations are shown. Data from triplicate independent experiments are described as the means ± SD. The p values were calculated using a Mann–Whitney U test.

the presence of patients' sera (data not shown). This result may not completely exclude the possibility that the autoantibody has some neutralizing activity, because fairly high concentration of rhIL-17F (50 ng/ml) was required to induce significant IL-6 production from fibroblasts. Future studies are necessary to distinguish these possibilities.

We further addressed profiles of autoantibodies in the 17 patients with STAT1 GOF (Table I). Patients with anti–IL-17F autoantibody were more likely to have anti-nuclear Ab (ANA) or other autoantibodies, although information of more patients is needed.

Discussion

In this study we reported two novel heterozygous *STAT1* GOF mutations of K278E in CCD and G384D in DBD that are responsible for CMCD. These mutations were associated with increased STAT1 phosphorylation due to impaired dephosphorylation as observed in the previous reports (4, 13).

Extensive analyses of CMCD patients resulting from impaired Th17 immunity such as IL-17F deficiency or IL-17RA deficiency have indicated nonredundant roles of Th17 cells and Th17-associated cytokines in host defense against mucocutaneous *Candida* infection (1). The development of CMCD in patients with heterozygous *STAT1* GOF mutations was also indicated to be associated with impaired differentiation of Th17 cells (3, 4, 13, 14). However, a CMCD patient with a heterozygous *STAT1* GOF mutation of L163R was recently shown to have normal CD4$^+$IL-17A$^+$ cells, although in vitro Th17 differentiation was impaired (7). Our study also demonstrated that CD4$^+$IL-17A$^+$ cells were not remarkably reduced at least in patient 2 with a heterozygous *STAT1* GOF mutation of G384D (Fig. 4). These findings prompted us to speculate that susceptibility to CMCD in patients with *STAT1*

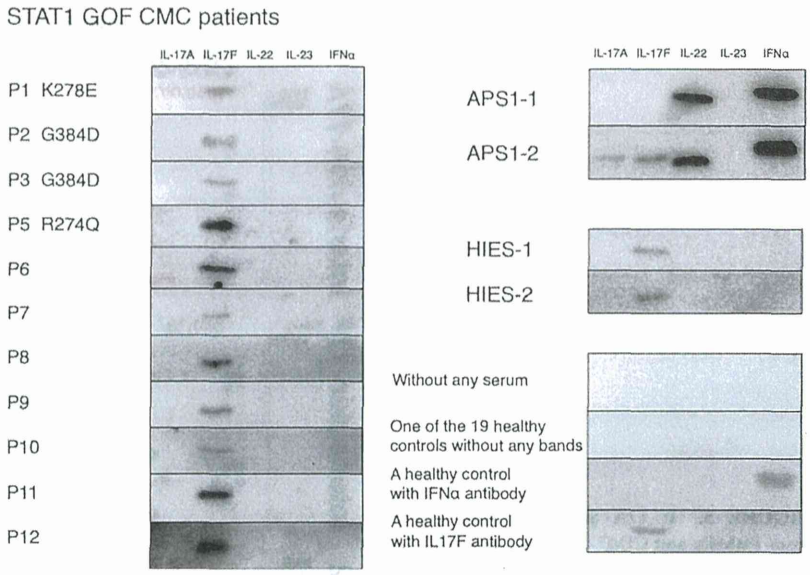

FIGURE 6. Immunoblot analysis showed the exclusive presence of anti–IL-17F autoantibody in the 11 of 17 STAT1 GOF patients' sera (64.7%). We confirmed the results by more than two independent experiments.

GOF mutations depends more on impaired production of other Th17-associated cytokines such as IL-17F or IL-22 than of IL-17A, which has been more extensively studied. ELISA and cytometric bead array studies unexpectedly revealed that production of IL-17F and IL-22 as well as IL-17A was not reduced in $CD4^+$ cells from the four patients with *STAT1* GOF mutations following PMA plus IOM stimulation. However, production of IL-17A and IL-22 but not IL-17F was significantly reduced in PBMCs or $CD4^+$ cells from these patients following more physiologically relevant stimulation of CD3/28. Therefore, it is possible that impaired production of IL-17A and IL-22 are more closely associated with the development of CMCD than of IL-17F in patients with *STAT1* GOF mutations in physiological conditions. Furthermore, production of IL-17A, IL-17F, and IL-22 from PBMCs was significantly reduced after *Candida* stimulation as observed in previous reports (3, 9), indicating that *Candida*-specific Th17 responses are impaired in patients with *STAT1* GOF mutations.

Whereas Th17 cells are widely accepted as the major IL-22 producers in the murine system (19–21), Th22 cells are suggested to be the major T cell subsets producing IL-22 in humans (22–24). Th22 cells are characterized by production of IL-22 with little or no IL-17, and they are important contributors to mucosal host defense. Thus, it is reasonable to think that deficient production of IL-22 from $CD4^+$ cells in the present patients was attributable to impaired Th22 differentiation and/or response, although it was not confirmed in this study.

Moreover, subsets of innate lymphoid cells (ILCs) recently identified in mice and humans are restricted to mucosal tissues and react promptly to acute bacterial or fungal infection (25). IL-17–producing ILC3, including lymphoid tissue inducer cells, produces IL-17 and IL-22, and IL-22–producing ILC3 produces only IL-22 (26). Recent studies indicate that ILC3 provides a rapid source of Th17-associated cytokines that is essential for early host protection, and Th17 or Th22 cells in turn become the dominant source of these cytokines that is required for complete clearance

Table I. Profiles of autoantibodies in the 17 patients with STAT1 GOF

Patient	Positive Autoantibodies	Negative Autoantibodies	αIL17F
1	ANA 160×, microsome 100×, TRAb 1.0 (< 0.9 IU/l)	TgAb	+
2		ANA, dsDNA, TRAb, TPO, TgAb, GAD, IAA, direct Coombs test	+
3	dsDNA 10 (<10 IU/ml)	ANA, TRAb, TPO, TgAb, GAD, IAA, direct Coombs test	+
4	TRAb (blocking rate 16% [<15%])	ANA	−
5	TgAb 55.33 (<40 IU/ml)	ANA, dsDNA, TRAb, TPO, GAD, IAA, IA-2, CCP, RF, c-ANCA, p-ANCA, AMA, αPL	+
6	ANA 160×	TPO, TgAb	+
7	Direct and indirect Coombs test	ANA, TRAb	+
8	ANA 40×	TPO, TgAb	+
9	ANA 40×	dsDNA, TPO, TgAb, GAD, IAA, IA-2	+
10	ANA 40×	dsDNA, TPO, TgAb	+
11	ANA 80×	TRAb	+
12		ANA, GAD, IAA, IA-2	+
13		ANA, TPO, TgAb	−
14		ANA, TRAb	−
15		ANA, TPO, TgAb, ASMA	−
16		ANA, TPO, TgAb	−
17		ANA	−

AMA, anti-mitochondrial Ab; ASMA, anti–smooth muscle Ab; c-ANCA, proteinase 3–anti-neutrophil cytoplasmic Ab; CCP, anti–cyclic citrullinated peptide Ab; dsDNA, anti-dsDNA Ab; GAD, anti–glutamic acid decarboxylase Ab; IA-2, anti-insulinoma–associated protein-2 Ab; IAA, insulin autoantibody; αIL17F, anti–IL-17F autoantibody; microsome, anti-microsomal Ab; p-ANCA, myeloperoxidase–anti-neutrophil cytoplasmic Ab; αPL, anti-phospholipid Ab; RF, rheumatoid factor; TgAb, anti-thyroglobulin Ab; TPO, anti–thyroid peroxidase Ab; TRAb, TSH receptor Ab.

of infection (26). Although genetic causes of CMCD have been associated with defects of Th17 immunity, they may also affect innate sources of IL-17 or IL-22. Future studies may provide an understanding of the relative contribution of innate and adaptive sources of these cytokines to the development of CMCD.

We further studied autoantibodies against Th17-associated cytokines in 17 patients with *STAT1* GOF mutations that could be associated with the development of CMCD. Autoantibodies were detected exclusively against IL-17F in sera from 11 of 17 patients and additionally 2 HIES patients (Fig. 6), although no neutralizing activity was observed. This result is in contrast with the previous report by Liu et al. (4) addressing that no autoantibodies against IL-17A, IL-17F, and IL-22 were detected in sera from patients with *STAT1* GOF mutations. The discrepancy may reflect the sensitivity of each assay system used, although the method of the previous study was not shown. One recent case report using immunoblot analysis showed the presence of anti–IL-17F autoantibody in a patient with R274Q mutation of *STAT1* (27). These results may indicate that anti–IL-17F autoantibody is useful as a marker for CMC, although the presence of this autoantibody may not be associated with the development of CMC.

Note that the expression of STAT1 GOF mutants was associated with impaired dephosphorylation of the endogenous WT STAT1 as well as the mutants themselves following the IFNs stimulation (Fig. 3). To our knowledge, this finding has not been reported because U3C cells deficient for endogenous STAT1 expression were used in the previous studies. Our results indicate that impaired dephosphorylation is present in heterodimers of WT STAT1 and GOF mutants as well as homodimers of the GOF mutants.

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Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL RESEARCH

Interstitial Lung Disease with Multiple Micrograniulomas in Chronic Granulomatous Disease

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Abstract
Background Chronic granulomatous disease (CGD) is a primary immunodeficiency disease that is characterized by susceptibility to bacterial and fungal infections. CGD patients also suffer from immune regulatory disorders, such as CGD-associated bowel inflammation with granuloma, which could be caused by excessive inflammation without demonstrable infection.
Purpose We investigated the clinical manifestation of interstitial lung disease (ILD) resulting from excessive inflammation in X-linked CGD patients.
Methods Pulmonary CT images and testing of serum KL-6 levels were performed to assess ILD in the patients. For this study, patients with pulmonary lesions due to demonstrable infections were excluded from among ILD patients.
Results Among 33 CGD patients, four developed ILD; they had increased reticulo-nodular opacities on CT images and elevated serum KL-6 levels. Histopathological examinations revealed multiple homogeneous micrograniulomas in the lesions of inflammatory cell infiltration. Mononuclear cells obtained from their pulmonary lesions produced higher amounts of inflammatory cytokines than the peripheral blood mononuclear cells of CGD patients, suggesting that the only infiltrating cells in the pulmonary lesions were activated and produced large amounts of inflammatory cytokines in ILD patients. Interestingly, an anti-inflammatory drug, such as a corticosteroid or thalidomide, but not anti-bacterial or anti-fungal drugs, improved CT image findings and reduced their KL-6 levels.
Conclusions CGD patients' daily exposures to inhaled antigens may induce excessive reactions with the production of inflammatory cytokines leading to the development of ILD with multiple micrograniulomas, which could be due to an inadequate production of reactive oxygen species in CGD.

Keywords Chronic granulomatous disease · interstitial lung disease · inflammation · granuloma · hypersensitivity pneumonia

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Introduction

Chronic granulomatous disease (CGD) is one of the primary immunodeficiency diseases that is characterized by inadequate production of reactive oxygen species (ROS) due to mutations of the genes that encode for the NADPH oxidase complex (NOX). As a result, CGD patients suffer from severe infections caused by pathogenic microorganisms, such as catalase-positive bacteria, mycobacterium, and fungi [1, 2].

Although progress in medical treatments, including new antibiotics and antifungal drugs, provides for infection control [3], other clinical manifestation of CGD has become problematic in these situations; namely, chronic hyperinflammation such as granuloma formation, CGD-associated bowel inflammation (CGD colitis) [4], and autoimmune disorders [5].

Although the mechanisms underlying this hyperinflammation are still under investigation, a plausible explanation is that the reduced ROS generated by impaired NOX function cannot adequately inhibit the production of inflammatory cytokines [6], and that this ROS deficit in CGD allows for the continuous production of inflammatory cytokines [7, 8], resulting in immune dysregulation or hyperinflammation. Thus, one of the effective therapeutic approaches for such hyperinflammation is the use of corticosteroids or immunosuppressive drugs [9].

Indeed, infliximab, a chimeric antibody against tumor necrosis factor-α (TNFα), has shown therapeutic efficacy for refractory CGD colitis, as TNFα is thought to play a critical role in granuloma formation in CGD. However, the use of these drugs increases a patient's susceptibility to pathogenic infections [10].

In this paper, we describe four patients with X-linked CGD who developed interstitial lung disease (ILD). These patients had elevated serum KL-6 levels and showed increased reticulo-nodular opacities on pulmonary CT images. Although their histopathological findings were reminiscent of those seen with hypersensitivity pneumonia (HP), treatment with allergen avoidance alone did not provide complete therapeutic effects for the clinical symptoms. On the other hand, anti-inflammatory drugs such as a corticosteroid or thalidomide did mitigate the clinical symptoms. Hence, ILD observed in these CGD patients was likely to be caused by excessive allergic reactions against non-pathogenic antigens.

Materials and Methods

Patients

All procedures and experiments were done after receiving informed consent from the patients or their parents. Our study protocol was approved by the Institutional Review Board of the National Center for Child Health and Development. In our hospital, there had been 33 patients with X-linked CGD confirmed by gene sequence analysis during the past 10 years. Four patients were confirmed to have developed ILD based on pulmonary CT images and elevated serum KL-6 level [11]. Among the ILD patients, those with pulmonary lesions due to demonstrable infections were excluded from this study.

Measurement of Specific IgG

Specific IgG antibody to *Aspergillus fumigatus* was determined in the serum of CGD patients (n=21; age, 19.0±10.1 year-old) including four ILD patients, and healthy volunteers (n=23; age, 17.6±9.1 year-old) using *A. fumigatus* IgG enzyme-linked immunosorbent assay kit (IBL, Hamburg, Germany).

Measurements of Cytokines in Serum and Bronchoalveolar Lavage Fluid

Serum levels of interleukin (IL)-6, IL-8, tumor necrosis factor-α (TNFα), and interferon-γ (IFNγ) were determined with a quantitative multiplex Milliplex system (Millipore, Billerica, MA) for CGD patients with ILD, X-linked CGD patients without demonstrable infections (n=10; age, 19.3±9.7 year-old), and healthy volunteers (n=10; age, 25.4±10.3 year-old). None of the CGD patients suffered from demonstrable infections. However, as they had previous pulmonary infections caused by bacteria or fungi, residual pathogens may remain due to elevated serum levels of βD-glucan (7.9±6.1 pg/ml; Normal range <10 pg/ml), thought to be a marker of fungal infection [12, 13]. Bronchoalveolar lavage (BAL) was performed during fiberoptic bronchoscopy under local anesthesia. Cytokine concentrations in BAL fluid (BALF) were also determined by Milliplex.

Lymphocyte Subset Analysis in Bronchoalveolar Lavage Fluid

Cells in BALF were characterized by flow cytometry (FACSAria; Becton, Dickinson and Company) using anti-human CD3, CD4, and CD8 monoclonal antibodies conjugated with allophycocyanin, phycoerythrin-Cy7, or peridinin–chlorophyll proteins-Cy5.5 (BioLegend, San Diego, CA).

Cytokine Production

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of CGD patients by density gradient centrifugation. Infiltrating cells in the lung were obtained from centrifuging BALF. The concentrations of IL-6, IL-8, TNFα, and IFNγ were determined for 2×10^6 cells/ml PBMCs and lung infiltrating cells without any stimulation in RPMI containing penicillin/streptomycin and 5 % human serum (Sigma-Aldrich) [14]. Cells were incubated for 16 h and cytokines in culture supernatants were determined by Milliplex.

Statistical Analysis

Experimental data were reported as the mean±standard deviation (s.d.). Group comparisons were made by Mann–Whitney tests, where appropriate, with Prism software (GraphPad Software, La Jolla, CA). Error bars indicate mean±s.d. A p-value of <0.05 was considered significant.

Results

CGD Patients Developed ILD with Increasing Serum Levels of KL-6

Among 33 patients with X-CGD who were followed at our hospital during the last 10 years, four (12 %) developed ILD during prophylactic treatment, as shown in Table 1. Two patients (Cases 1 and 3) showed mild restrictive ventilatory impairments by respiratory function testing (Table 1). As with other types of ILD, the serum levels of KL-6, thought to be a sensitive marker for ILD [11], increased to 1,030–4,000 IU/ml for these four patients at the onset of ILD. Conversely, serum levels of KL-6 were in the normal range (<500 IU/ml) for CGD patients without ILD (311±106 IU/ml; Fig. 1a and b). Although no pathogenic microorganisms were isolated from their blood and sputum cultures, and *Pneumocystis jirovecii* and *Mycobacterium* spp. were not detected by PCR assay using their sputa at the onset of ILD, it is difficult to completely deny the impact of infection on ILD. Accordingly, prophylactic treatment with itraconazole and trimethoprim/sulfamethoxazole was required in the ILD patients. Antibiotics and antifungal drugs did not have any therapeutic effects on ILD symptoms. One patient (Case 4) received periodical subcutaneous injections of IFNγ along with the prophylactic drugs (Table 1). The other three patients with ILD did not receive IFNγ and just one (Case 4) of ten CGD patients who had been treated with this therapy developed ILD (odds ratio=0.74), suggesting that IFNγ was unlikely to have been involved in the development of ILD.

Clinical Courses of CGD Patients with ILD

Case 1: The patient was a 20-year-old Japanese man who had been treated with 7.5 mg/day of corticosteroid for CGD-associated bowel inflammation. Just after working at a fruit-processing plant, he developed a cough which became persistent at 8 weeks. He subsequently quit this job to avoid breathing in the dust at the plant where he worked. Leaving the job improved his clinical symptoms and increased his oxygen saturation from 90 to 98 %. However, lung CT images still showed diffuse ground-glass opacity (Fig. 2a) and serum KL-6 levels remained high (Fig. 1b). An oral corticosteroid was increased to 40 mg/day at 12 weeks after the onset of ILD. Although the increased corticosteroid resulted in some improvements of ILD on CT images, this therapy worsened his pulmonary fungus infection. Thus, the dose of this drug was reduced and thalidomide therapy was started at 38 weeks after the onset of ILD. Subsequently, his clinical condition, including CT findings, was relatively stable and his serum KL-6 levels gradually decreased with corticosteroid and thalidomide therapy (Fig. 1b).

Case 2: The patient was an 8-year-old Japanese boy. ILD was serendipitously identified by CT images acquired for follow-up of lung abscesses he had since 4 years old (Fig. 2b). As the nodular consolidation of CT images did not improve after more than one antibiotic or antifungal drug use, he was treated with 0.5 mg/kg/day of a corticosteroid at 8 weeks after the onset of ILD. His serum KL-6 levels declined after this therapy (Fig. 1b), but increased again with gradual tapering of the corticosteroid dose.

Table 1 Characteristics of the CGD patients with ILD

Case	Age at diagnosis of ILD (year-old)	Prophylactic treatment			Respiratory signs and symptoms			Lung function	
		IFNγ (JRU/m^2/week)	Trimethoprim / Sulfamethoxazole (g/kg/day)	Itraconazole (mg/kg/day)	Crackle	Cough	Fever	FEV1.0%[a]	%VC[b]
1	20	–	0.07	4.4	+/–	+	+	86.2	72.5
2	8	–	0.06	5.0	–	–	–	81.1	103.4
3	23	–	0.08	4.0	–	+	+	73.1	72.8
4	8	25×10^4	0.06	5.0	–	–	–	93	75.6

[a] FEV1.0 %, forced expiratory volume 1.0 s %; Normal range >70

[b] 2 %VC, % vital capacity; Normal range >80

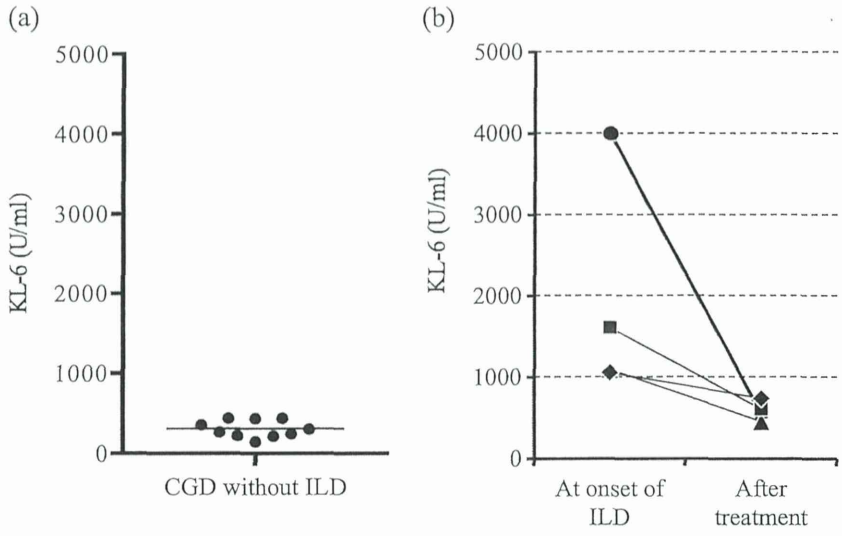

Fig. 1 Serum KL-6 levels of CGD patients with and without ILD. **a** Serum KL-6 levels were determined for CGD patients who previously had pulmonary infections caused by bacteria or fungi (311±106 IU/ml; n=10). **b** Serum KL-6 levels of Case 1 (*circles*), Case 2 (*triangles*), Case 3 (*squares*) and Case 4(*diamonds*) at the onset of ILD and after treatment

Case 3: The patient was a 23-year-old Japanese man. After moving to a new residence, he developed a persistent cough and a prolonged fever for 2 months despite the administration of antibiotics and antifungal drugs at our hospital. He had high KL-6 serum levels (Fig. 1b) and CT images showed diffuse ground-glass opacity at the onset of ILD (Fig. 2c). He moved back to his previous residence as an allergen avoidance measure. This resulted in a decline of his serum KL-6 levels and improvements on CT images without any medications (Fig. 1b).

Case 4: The patient was an 8-year-old Japanese boy who received periodical subcutaneous injections of IFNγ together with prophylactic drugs (Table 1). He was scheduled to receive thalidomide therapy for CGD colitis. A pulmonary CT scan taken as a screening test before thalidomide therapy revealed an ILD lesion (Fig. 2d) and an elevated serum KL-6 level (Fig. 1b). Thalidomide therapy resulted in a decline of his serum KL-6 levels; however, the levels increased again upon discontinuation of therapy (Fig. 1b).

Pathological Findings for ILD Patients Revealed Inflammation of Interstitial Lung Tissue

To assess the pathological changes in the lung lesions of the ILD patients, we collected BALF by bronchoscopy for Cases

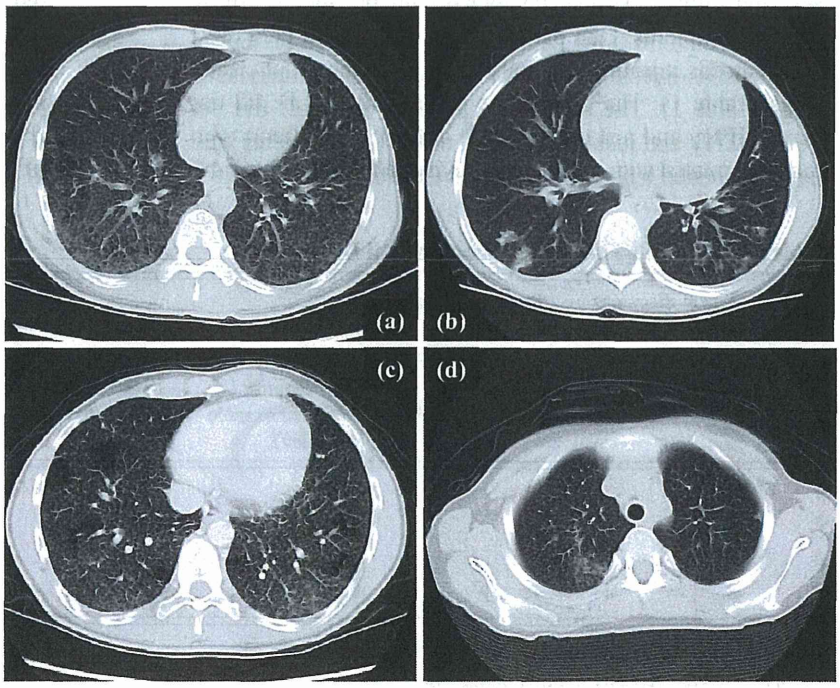

Fig. 2 Computed tomographic lung images at the onset of ILD. Representative chest CT images revealed ground glass opacity for Case 1 (**a**), Case 2(**c**), Case 3(**c**) and Case 4 (**d**), and nodular consolidation for Case 2 (**b**) at the onset of ILD

1, 2, and 3. Although the number of cells in BALF varied among these cases, the main fractions were lymphocytes. Conversely, in CGD patients with pulmonary aspergillosis, the fractions were predominantly macrophages (n=3 for CGD patients with aspergillosis, Table 2). Flow cytometry analyses revealed that most lymphocytes in BALF were $CD3^+$ T cells, and the ratio of $CD4^+$ to $CD8^+$ T cells was less than 1.0 for Cases 1 and 3, which suggested that most lymphocytes were $CD3^+CD8^+$ cytotoxic T cells in these patients with diffuse pulmonary lesions (Table 2).

Lung tissue samples were obtained from Cases 1 and 3 by surgical lung biopsy and examined after hematoxylin and eosin staining to assess pathological changes in these lesions. Homogeneous microgranuloma formation surrounded by the infiltration of multi-nucleated giant cells and lymphocytes were revealed, which was reminiscent of those seen with HP (Fig. 3a and b). Since blood and lymphatic vessels were not occupied by granulomas, and that proteinase three anti-neutrophil cytoplasmic antibodies (PR-3ANCA) in serum were negative for these cases, the likelihood of sarcoidosis and Wegener's granulomatosis was low. As well, no pathogenic microorganisms were isolated from BALF and lung tissues.

High Levels of Specific IgG to *Aspergillus fumigatus* in Serum of CGD Patients

Pulmonary aspergillosis can be distinguished into two types; infection and hypersensitivity respiratory disorders including HP that is caused by a prototypical type-III and type-IV allergic inflammatory reaction [15]. In order to assess the exposure to *Aspergillus* spp. in CGD patients, we determined serum levels of specific IgG antibodies against *A. fumigatus*, a common genus in living environment. The concentration of specific IgG to *A. fumigatus* in the serum of CGD patients was significantly higher than that of healthy subjects, while there was no difference between CGD patients with and without ILD (108.3±7.5 U/ml, 143.4±64.3 U/ml and 12.5±16.6 U/ml for CGD patients with and without ILD and healthy subjects, respectively, Fig. 4).

Infiltrating Cells in ILD Patients' Pulmonary Lesions were Activated and Produce Large Amounts of Inflammatory Cytokines

To assess whether inflammatory cytokines, including IL-6, IL-8, TNFα, and IFNγ, were involved in the pathogenic changes in ILD pulmonary lesions, we measured these cytokines in BALF samples. Interestingly, the levels of these cytokines in BALF samples from ILD patients were much higher than those of CGD patients with pulmonary aspergillosis (Fig. 5a). By comparison, the serum levels of these cytokines for ILD patients fell between those of CGD patients without demonstrable infections and healthy subjects (Fig. 5b). This suggested that in ILD patients, PBMCs were not activated in the peripheral blood but in the pulmonary lesions instead (Fig. 5b). This was also confirmed by an in vitro cytokine production assay whereby the amounts of these inflammatory cytokines produced by PBMCs and cells obtained from BALF were measured.

There were no differences in the serum levels of the inflammatory cytokines between ILD patients and CGD patients without demonstrable infections. Hence, circulating PBMCs should have been similarly activated in both of these groups. When cultured under conditions of no stimulation, cells obtained from BALF samples for Cases 1 and 2 produced higher amounts of these cytokines than the unstimulated PBMCs from CGD patients (Fig. 6). This suggested that only infiltrating cells in the pulmonary lesions were activated and produced large amounts of inflammatory cytokines in ILD patients.

Discussion

Since CGD patients have susceptibility to infection, some residual pathogens may have persisted and invaded their lungs partially. In our cases, the initial symptoms of ILD might overlap with residual pathogen and microgranuloma formation due to hyperinflammation; however, increased levels of

Table 2 Assessments of ILD patients' bronchoalveolar lavage fluids

	Cellularity (10^5 cells/ml)	Cell differentiation (%)				Lymphocyte (%)			CD4/CD8
		Macrophage	Lymphocyte	Eosinophil	Neutrophil	CD3	CD4	CD8	
Case 1	10.1	34	66	0	0	98	39.9	47.2	0.8
Case 2	3.2	24	75.5	0.5	0	82	57.4	24.4	2.4
Case 3	19	23.8	73.4	0.2	2.2	98.9	40.6	56.4	0.7
Aspergillosis	5.3±6.3	78.2±11.1	20.2±9.7	0.3±0.6	1.3±1.2	89.9±4.4	63.3±11.1	26.1±5.1	2.5±0.9
Normal range	0.2–1.0	75–95	4.0–25	<1.0	–	–	33–57	14–28	1.5–3.2

Aspergillosis, CGD patients who suffer from pulmonary aspergillosis (n=3)

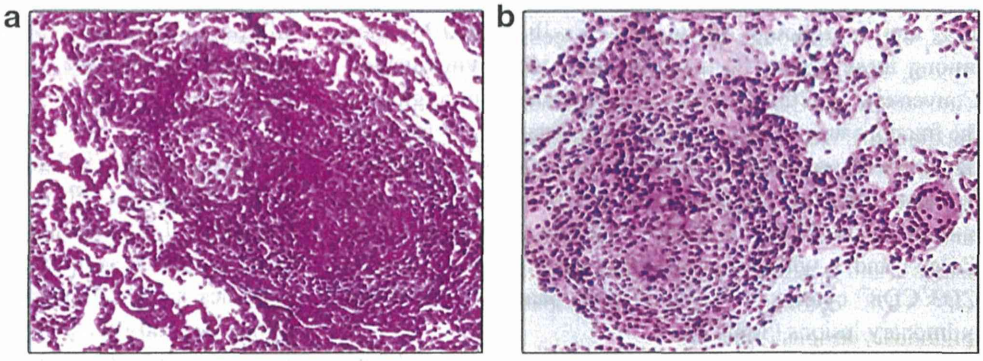

Fig. 3 Pathological findings reveal homogeneous microgranulomas formation in ILD patients' lungs. Pathologic evaluations showed infiltrations of inflammatory cells and homogeneous formations of microgranulomas on pulmonary sections stained with hematoxylin and eosin at the onset of ILD for Case 1 (**a**) and Case 3 (**b**)

serum KL-6 and failure of adequate therapy directed at bacterial and fungal infection could lead to consideration of other etiologies such as hyperinflammation in CGD. Meanwhile, there is a previous report of a CGD patient who developed interstitial inflammation of lung resulting from hyperinflammation associated with CGD [16].

HP is a pulmonary interstitial inflammatory disease caused by type-III and type-IV allergic inflammatory reaction to more than 300 inhalation allergens, including *Aspergillus* spp. [15, 17]. The specific IgG to *Aspergillus* was increased in serum of CGD patients, which was also observed in patients with HP [18]. Based on the disease duration, HP is categorized as acute, sub-acute, or chronic [19]. As there is no demonstrable evidence of progressive infection and the clinical course, including pathological findings, that are similar to those of HP, the mechanisms of ILD in these cases may be associated with HP. If so, the incidence of ILD is probably much higher in CGD patients because the frequency of HP is four per million children [20]. In our study, we identified four ILD patients out of 33 X-CGD patients (12 %). It is intriguing that there was a discrepancy between clinical phenotypes and pathological findings, in that the clinical courses of our ILD patients were reminiscent of sub-acute HP, whereas the pathological findings of homogeneous microgranulomas reflected typical acute HP.

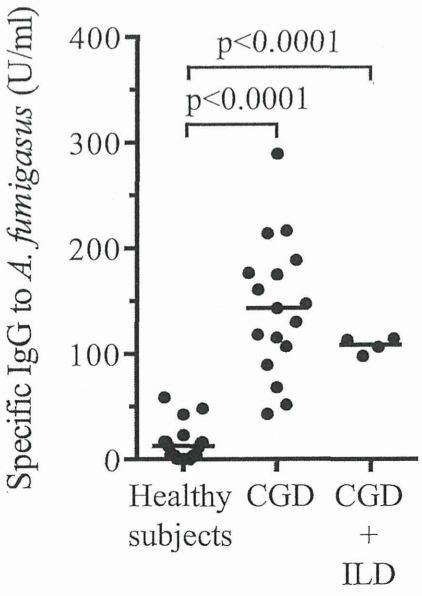

Fig. 4 Specific IgG antibody to *Aspergillus fumigatus* in CGD patients with and without ILD. Specific IgG antibody to *Aspergillus fumigatus* in serum was increased in CGD patients with and without ILD compared to that of healthy subjects

The involvement of IFNγ therapy with the development of ILD cannot be ruled out completely. However, because three of our patients who did not receive IFNγ therapy developed ILD, this may undermine the possibility. In particular, the clinical symptoms in two patients (Cases 1 and 3) were mitigated only by allergen avoidance, proving that CGD patients were more susceptible to ILD triggered by prolonged hyperinflammation resulting from inhalation antigens.

Recently, the clinical symptoms of auto-inflammation, such as granuloma formation or CGD colitis, were reported in CGD [7, 4]. A plausible explanation is that a deficit of ROS generation due to impaired NOX function in CGD patients prolongs NF-κB activation and caspsase-1 deactivation, which results in hyperinflammation [6]. This is because ROS are negative regulators for inflammatory cytokine production through the ERK, NF-κB, and caspsase-1 signaling pathway [8, 21]. In keeping with this, the PBMCs of CGD patients would be activated to produce inflammatory cytokines due to the effects of remaining pathogens, even in a static state [7]. Importantly, cells in BALF samples were activated to produce large amounts of inflammatory cytokines compared to the PBMCs of CGD patients, suggesting that the sites of inflammation were localized to the lungs due to inhalation of antigens. Previous reports of p47phox and gp91phox-deficient mice developing exaggerated progressive lung inflammation following inhalation of zymosan or LPS may provide a basis for our findings [22]. Zymosan is a fungal wall component that induces an innate immune response [23]. Elevated specific IgG antibody to *A. fumigatus* in CGD patients suggests that the patients are repeatedly exposed to an *Aspergillus* component which activates alveolar macrophages [24].

While avoidance of allergen exposure, such as relocating or changing jobs, is the initial therapy for HP, this measure alone