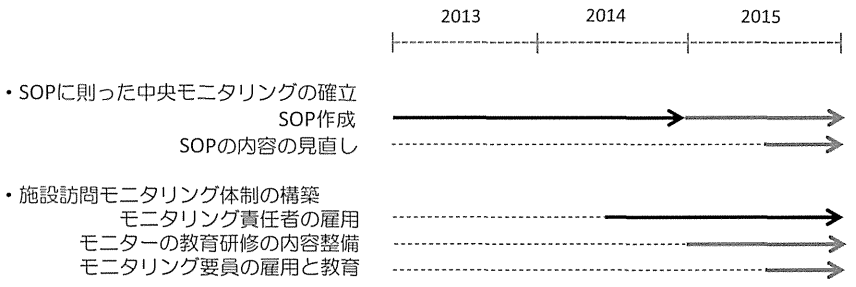
モニタリング体制の整備ロードマップ



ひきつづき各種SOPの作成を進めるとともに、モニターを雇用して教育することによって、施設訪問によるSDVモニタリングを実施する体制を充実させる.
さらに実際の遺伝子治療の経験に基づいて、SOPの内容の見直しを行う.

Ⅳ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅴ．　研究成果の印刷物・別刷

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BRIEF COMMUNICATION

Augmentation of antitubercular therapy with IFNγ in a patient with dominant partial IFNγ receptor 1 deficiency

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KEYWORDS
Mendelian susceptibility to mycobacterial diseases;
Interferon-γ receptor 1 deficiency;
Mycobacterium bovis Bacille Calmette–Guerin;
Osteomyelitis;
Interferon-γ

Abstract Osteomyelitis due to *Mycobacterium bovis* Bacille Calmette–Guerin (BCG) often develops in patients with interferon-γ receptor 1 (IFNγR1) deficiency. In these patients, susceptibility appears to be caused by impaired interleukin-12- and IFNγ-mediated immunity. Here we report the case of a one-year-old girl with dominant partial IFNγR1 deficiency who suffered from lymphadenitis and multiple sites of osteomyelitis due to BCG infection. She was allergic to isoniazid and rifampicin – the prescribed standard treatment – and required prior desensitization therapy. She was subsequently treated with these drugs, but her symptoms did not improve. IFNγ therapy was added to the antitubercular therapy, increasing the serum level of IFNγ and leading to the resolution of the lymphadenitis and osteomyelitis. In conclusion, high dose IFNγ therapy in combination with antitubercular drugs led to resolution of BCG infection in a patient with dominant partial IFNγ deficiency.

1. Introduction

Mendelian susceptibility to mycobacterial disease (MSMD) is a rare primary immunodeficiency characterized by a deficiency in the interleukin (IL)-12/23–interferon-γ (IFNγ)

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axis. Type-1 cytokine response is crucial for human host defense against intracellular pathogens. Patients with MSMD demonstrate increased susceptibility to infections of environmental non-tuberculous mycobacteria, *Salmonella* and *Mycobacterium bovis* (*M. bovis*) Bacille Calmette–Guerin (BCG) [1,2]. Several genetic mutations have been identified in patients with MSMD. Mutations have been found in genes coding for IL-12β, IL-12 receptor β1, IFNγ receptor 1 (IFNγR1), IFNγ receptor 2 (IFNγR2), signal transducers and activator of transcription (STAT1), NF-κB essential modulator (NEMO), gp91phox, tyrosine kinase 2 (TYK2), interferon regulatory factor 8 (IRF8) and interferon-stimulated gene 15 protein (ISG15). The 818del4 mutation of *IFNGR1* gene results in a truncated protein that exerts a dominant negative effect on the wild-type IFNγR1 molecule. Accumulation of truncated IFNγR1 proteins impedes the function of normal IFNγR1 molecules encoded by the wild-type allele [3,4], leading to a diminished cellular response to ligand binding. Previous reports have shown that BCG causes recurrent and refractory osteomyelitis in patients with dominant partial IFNγR1 deficiency [5–8]. Among vaccinated children, almost 70% of those with dominant partial IFNγR1 deficiency develop osteomyelitis due to BCG [1].

Standard therapy caused by *M. bovis*, recommended by the Centers for Disease Control and Prevention (CDC) and the American Academy of Pediatrics (AAP), is a multidrug regimen that includes isoniazid, rifampin and ethambutol (*M. bovis* is inherently resistant to pyrazinamide). However, in patients with dominant partial IFNγR1 deficiency develop osteomyelitis, BCG infection is relatively resistant to this type of therapy [1,6]. Moreover, allergy against such drugs is also present in some cases. Therefore, an alternative approach is necessary for such patients.

Here, we describe a Japanese girl with dominant partial IFNγR1 deficiency who suffered from BCG multiple sites of osteomyelitis and lymphadenitis. She was allergic to isoniazid and rifampicin but was treated with the drugs following desensitization therapy. Unfortunately, standard therapy did not resolve the infection. In light of her genetic background, IFNγ was added to her antitubercular drug regimen. The combination of isoniazid, rifampicin, ethambutol and high dose IFNγ successfully cured her multi-site osteomyelitis and lymphadenitis.

2. Patient and method

The patient, a one-year-old girl (vaccinated with BCG at 2 months of age), suffered from axillary lymphadenitis at 10 months of age. Three months later, she was presented with osteomyelitis at multiple sites, including the skull, humerus, tibia and cervical vertebra (Fig. 1a). Axillary lymph node and skull tissue biopsies revealed the presence of *M. bovis* BCG (BCG Tokyo 172 strain). Immunological assessment was performed to evaluate the presence of primary phagocytic disorders, such as chronic granulomatous disease [9] or MSMD. Flow cytometry revealed a five-fold increase in the expression of IFNγR1 on $CD14^+$ monocytes isolated from the patient (Fig. 1c) (mean fluorescence intensity of IFNγR1: 10371, 51350, and 50583 for a healthy subject, the patient, and the patient's mother, respectively). The diagnosis of dominant partial IFNγR1 deficiency was confirmed by a genetic analysis that revealed one four-nucleotide deletion in exon 6 of *IFNGR1* (818del4) and one wild-type *IFNGR1* allele (Fig. 1d). Interestingly, the patient's mother carried the same *IFNGR1* deletion and suffered from one episode of osteomyelitis and multiple subcutaneous abscesses due to *Mycobacterium* spp. at one year of age, though she did not receive BCG vaccination. The patient and her mother were thus diagnosed with inherited dominant partial IFNγR1 deficiency.

3. Results

3.1. Addition of high dose IFNγ to antitubercular regimen

The patient was initially treated with isoniazid and rifampicin, based on the guidelines of the CDC and AAP. However, she developed generalized erythema multiform exudativum two weeks after commencement of treatment. Consequently, her antitubercular drug regimen was changed to ethambutol and levofloxacin due to the antimicrobial sensitivity to *M. bovis* isolated from her osteomyelitis lesion. This regimen proved ineffective in treating her lymphadenitis and osteomyelitis. We decided to put her on a two-month course of desensitization to isoniazid and rifampicin according to the Guidelines of Japanese Society of Tuberculosis [10]. Desensitization of each drug started at 0.2 mg/kg/day and the dose was doubled every 7–10 days to reach 10 mg/kg/day (Fig. 1e). Although desensitization therapy was successful, the patient failed to improve with this medical regimen.

Upon obtaining informed consent from the family, IFNγ was added to the regimen. IFNγ was initially administered subcutaneously at 250 000 JRU/m^2 per week, a sufficient dose for infection prophylaxis in chronic granulomatous disease [11], and up-titrated thereafter. Treatment with IFNγ resulted in an increase in serum IFNγ level, and a decrease in serum IL-6 level (Fig. 1e) with no change in serum TNFα and IL-12 levels (although the patient's serum TNFα level was higher than that of healthy subjects at baseline: 16.5 ± 4.0 pg/ml vs. 1.1 ± 0.9 pg/ml, respectively, $p < 0.001$). A final dose of 1250 000 JRU/m^2 IFNγ per week led to resolution of her axillary lymphadenitis and ossification of the multiple osteomyelitic lesions (Fig. 1b).

3.2. Assessment of the effect of high dose IFNγ on the patient's immune function

Peripheral blood mononuclear cells (PBMCs) were isolated from the patient, the patient's mother and healthy subjects (n = 3), and stimulated with lipopolysaccharide (LPS) with or without IFNγ. The amount of TNFα produced by the stimulated PBMCs was measured by quantitative multiplex detection using Milliplex (Millipore, Billerica, MA). When PBMCs were stimulated with LPS alone, similar amounts of TNFα were produced (Fig. 2). In PBMCs isolated from healthy donors, the addition of IFNγ led to an increase in TNFα production in a dose-dependent manner; whereas in PBMCs isolated from the patient and her mother, TNFα production increased only when IFNγ was added at the maximum concentration (10^5 JRU/mL) (Fig. 2).

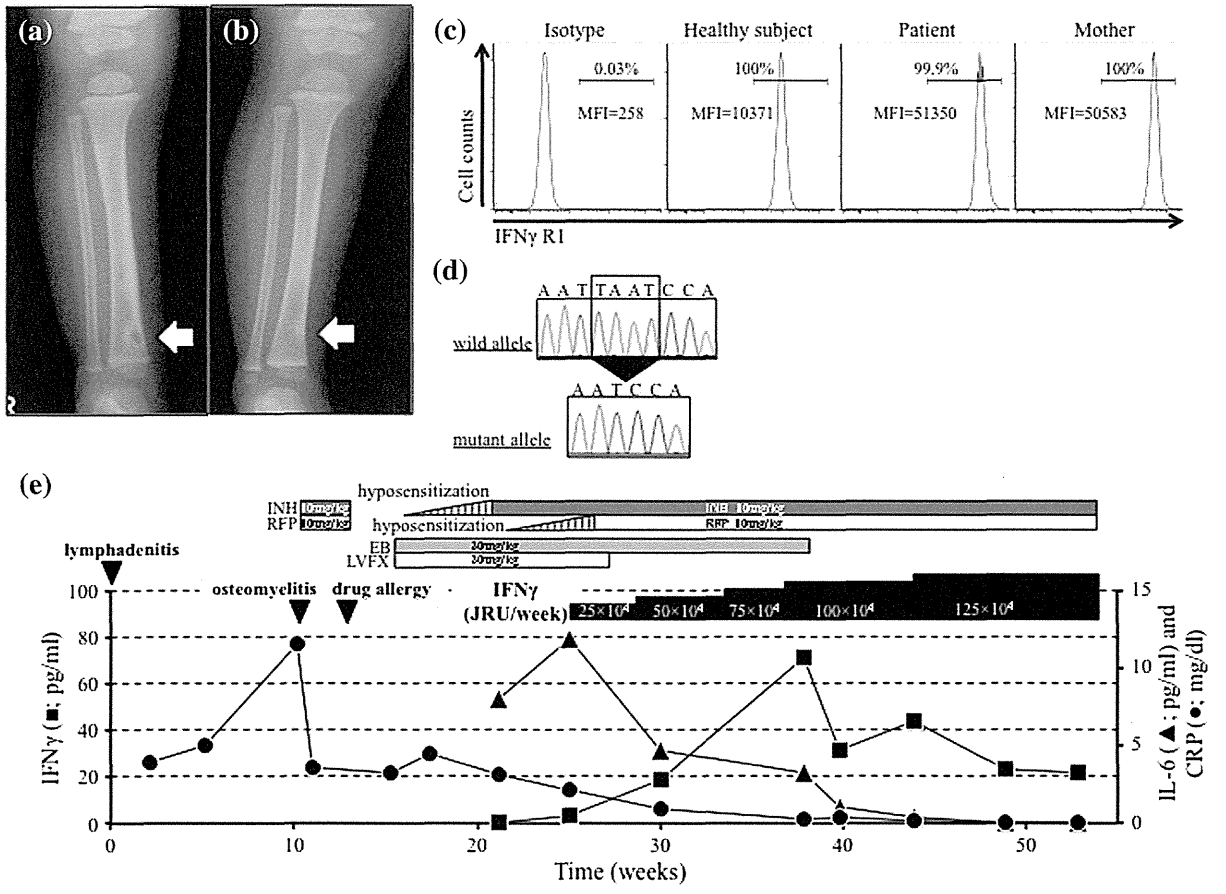

Figure. 1 Clinical course of BCG osteomyelitis in a patient with dominant partial IFNγ receptor 1 deficiency. (a)–(b) X-rays demonstrating osteomyelitis of the right tibia before IFNγ administration (a) and ossification of the lesion following IFNγ therapy (b). (c) Representative flow cytometry data. PBMCs were identified with FACSAria IIIu using anti-human CD14 and IFNγR1 monoclonal antibodies conjugated with allophycocyanin and phycoerythrin, respectively (BioLegend, San Diego, CA). MFI, mean fluorescence intensity. (d) Genetic analysis of the patient's genomic DNA revealed a 4-nucleotide deletion in exon 6 of *IFNGR1* (818del4) and a wild-type *IFNGR1* allele. (e) Clinical time course of treatment with IFNγ and multidrug antitubercular therapy. Serum levels of IFNγ (squares), IL-6 (triangles), and CRP (circles) were assessed during treatment.

4. Discussion

In the patients with dominant partial IFNγR1 deficiency, BCG infection is relatively resistant to conventional antitubercular therapy, although the clinical features of dominant partial IFNγR1 deficiency are less severe than those of complete deficiency [1,6]. Our data indicated that a high dose of IFNγ was capable of restoring the patient's impaired immune response to BCG infection. Superphysiologic IFNγ can overcome the dominant negative effect of truncated IFNγ by binding to the dimerized residual wild-type receptor, leading to the production of STAT1 associated-cytokines such as IFNγ, TNFα, IL-12 and IL-6 [7,12]. We found that in PBMCs from the patient and her mother, high dose IFNγ enhanced LPS-induced TNFα production.

Interestingly, PBMCs from the patient and her mother produced as much TNFα as healthy PBMCs in response to LPS alone. This suggests that the TLR4 signaling axis remains intact in PBMCs from patients with dominant partial IFNγR1 deficiency. It should be noted that serum TNFα levels were elevated in the patient but not in her mother, indicating residual BCG infection even in the context of normal serum IL-6 and C-reactive protein (CRP) levels. Collectively, these findings show that TNFα levels may be a useful measure of infection severity in patients with dominant partial IFNγR1 deficiency.

5. Conclusion

We have demonstrated that high dose IFNγ, when added to standard antitubercular regimen, is effective in the treatment of multi-site osteomyelitis and lymphadenitis in patients with dominant partial IFNγ deficiency.

Conflict of interest

The authors declare that they have no conflicts of interest.

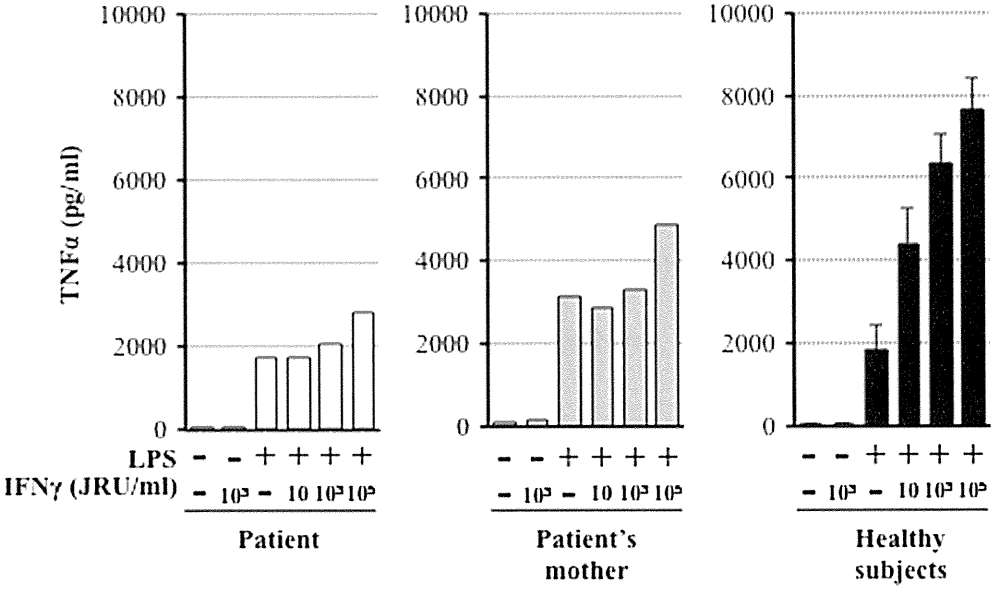

Figure. 2 TNFα production in response to LPS plus IFNγ in PBMCs. PBMCs isolated from the patient (white bars), patient's mother (gray bars), and healthy subjects (black bars) were stimulated with LPS alone or LPS plus serial concentration of IFNγ for 20 h. The level of TNFα in culture supernatant was measured by quantitative multiplex detection using Milliplex.

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ORIGINAL RESEARCH

Interstitial Lung Disease with Multiple Microgranulomas 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 microgranulomas 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 microgranulomas, 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

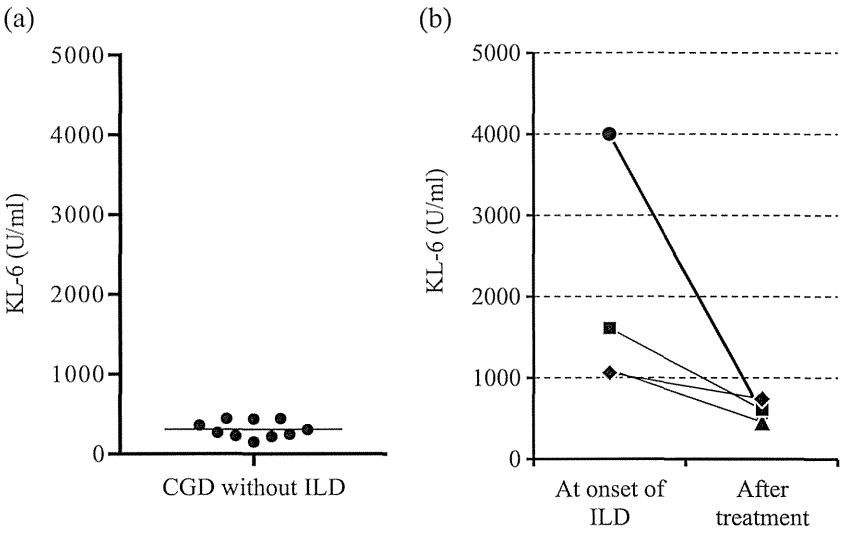

Fig. 1 Serum KL-6 levels of CGD patients with and without ILD. **a** Serum KL-6 levels were determined for CGD patients who previously had pulmonary infections caused by bacteria or fungi (311±106 IU/ml; $n=10$). **b** Serum KL-6 levels of Case 1 (*circles*), Case 2 (*triangles*), Case 3 (*squares*)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

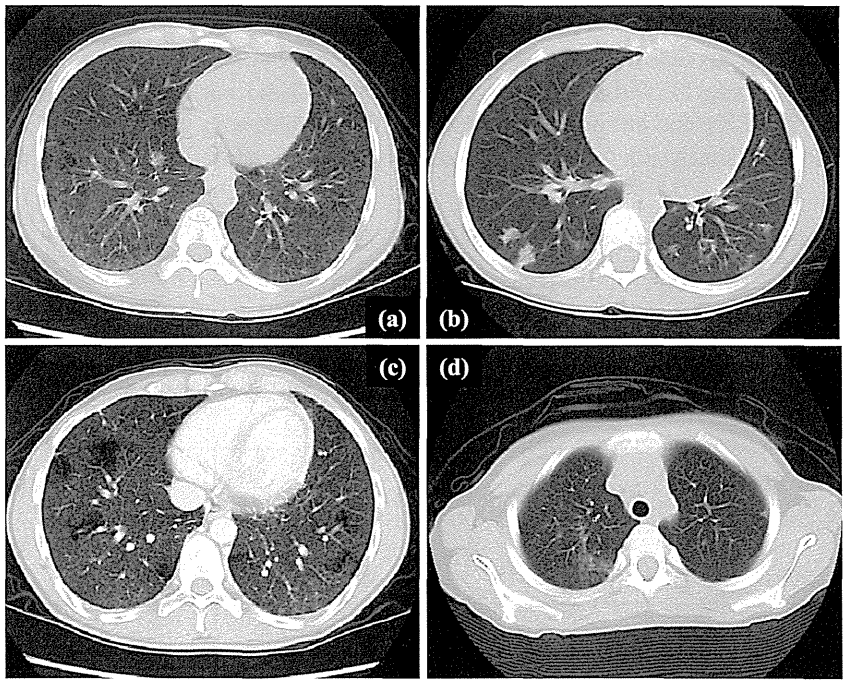

Fig. 2 Computed tomographic lung images at the onset of ILD. Representative chest CT images revealed ground glass opacity for Case 1 (**a**), Case 2(**c**), Case 3(**c**) and Case 4 (**d**), and nodular consolidation for Case 2 (**b**) at the onset of ILD

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)

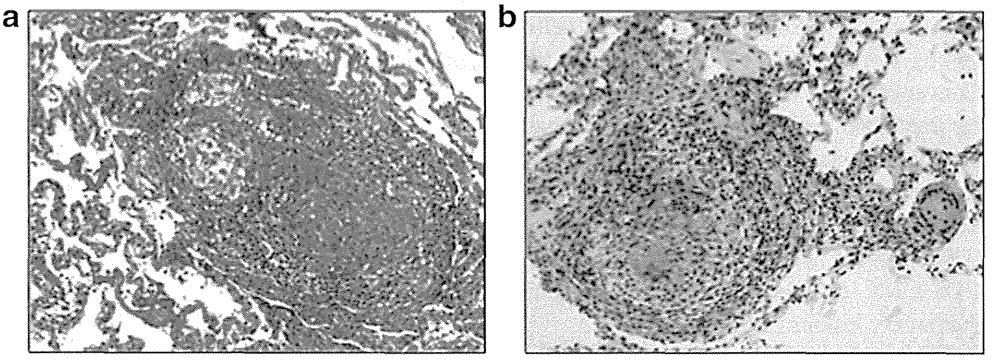

Fig. 3 Pathological findings reveal homogeneous microgranulomas formation in ILD patients' lungs. Pathologic evaluations showed infiltrations of inflammatory cells and homogeneous formations of microgranulomas on pulmonary sections stained with hematoxylin and eosin at the onset of ILD for Case 1 (**a**)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

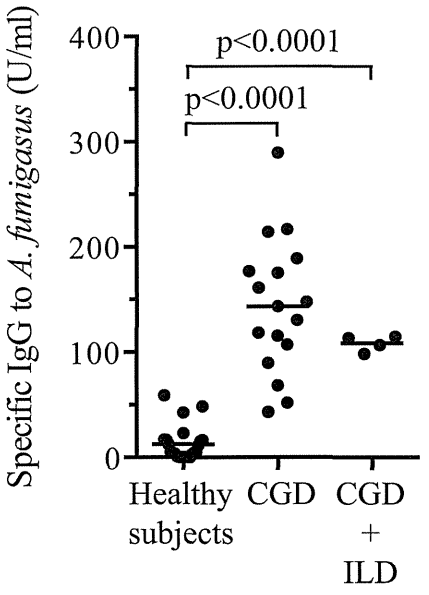

Fig. 4 Specific IgG antibody to *Aspergillus fumigatus* in CGD patients with and without ILD. Specific IgG antibody to *Aspergillus fumigatus* in serum was increased in CGD patients with and without ILD compared to that of healthy subjects

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

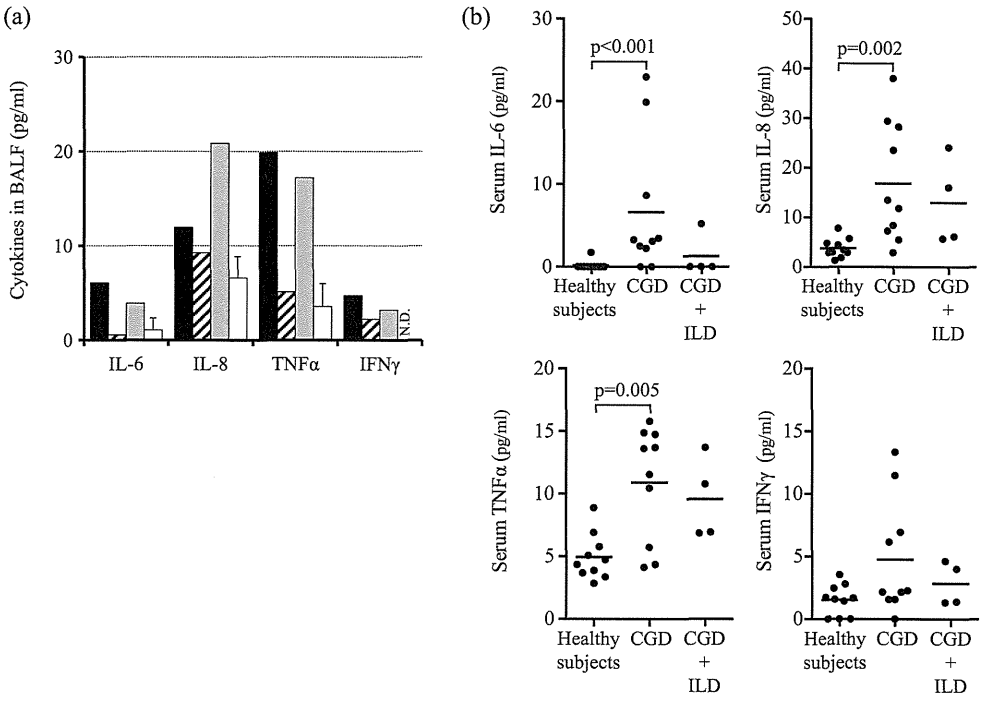

Fig. 5 Cytokine levels in bronchoalveolar lavage fluid and serum are increased in CGD patients. **a** BALF concentrations of IL-6, IL-8, TNFα, and IFNγ for Case 1 (*black bars*), Case 2 (*striped bars*), Case 3 (*gray bars*), and CGD patients who developed pulmonary aspergillosis (white bars, $n=3$). **b** Serum levels of IL-6, IL-8, TNFα, and IFNγ in ILD patients, CGD patients without demonstrable infection ($n=10$), and healthy subjects ($n=10$)

may be insufficient to provide ILD patients with complete therapeutic effects for their clinical symptoms, as their PBMCs lack negative regulators of ROS for inflammatory cytokine production. Meanwhile, anti-inflammatory therapy using steroid (e.g., oral corticosteroid) has been reported to be successful in controlling CGD colitis [25]. However, it should be noted that this therapy often increases a patient's susceptibility to infection [26], as was the situation in Case 1. From this perspective, thalidomide therapy should be considered for CGD patients with ILD because it potentially suppresses inflammation by decreasing inflammatory cytokine production through inhibition of NF-κB. This mode of action has been demonstrated in patients with Behcet's disease, rheumatoid arthritis, and Crohn's disease [27–30]. Also, thalidomide therapy exerts has a smaller negative effect on host defense [31]. Previously, we have reported that thalidomide attenuated excessive inflammation without increasing the susceptibility to infection in a patient with CGD colitis [7].

Conclusions

We described the clinical courses of 4 X-CGD patients with ILD and assessed the functions of PBMCs from CGD patients based on the cells' production of inflammatory cytokines. Although their pathological findings were reminiscent of HP,

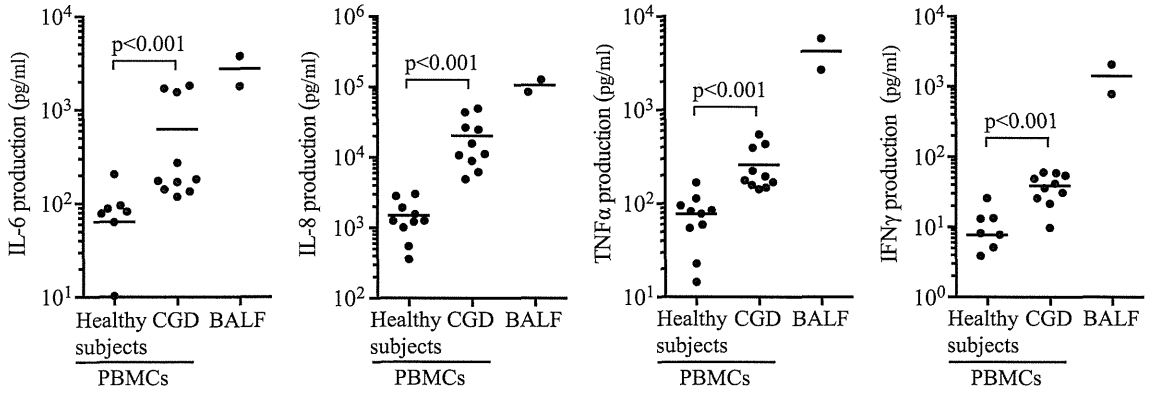

Fig. 6 Cytokine production by PBMCs and infiltrating cells in BALF. Cytokine levels were determined in culture supernatants after PBMCs or BALF cells were cultured for 16 h without stimulation. Concentrations of IL-6, IL-8, TNFα, and IFNγ in culture supernatants of infiltrating cells obtained from BALF and PBMCs obtained from CGD patients without demonstrable infection ($n=10$) and healthy subjects ($n=10$)