Two Novel Gain-of-Function Mutations of \textit{STAT1} Responsible for Chronic Mucocutaneous Candidiasis Disease: Impaired Production of IL-17A and IL-22, and the Presence of Anti–IL-17F Autoantibody

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\textit{J Immunol} published online 6 October 2014
http://www.jimmunol.org/content/early/2014/10/03/jimmunol.1401467

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Two Novel Gain-of-Function Mutations of STAT1 Responsible for Chronic Mucocutaneous Candidiasis Disease: Impaired Production of IL-17A and IL-22, and the Presence of Anti–IL-17F Autoantibody

Yasuhiro Yamazaki,* Masafumi Yamada,* Toshinao Kawai,† Tomohiro Morio,* Masafumi Onodera, ‡ Masahiro Ueki,* Nobuyuki Watanabe,* Hidetoshi Takada,§ Shunichiro Takezaki,* Natsuko Chida,*¥ Ichiro Kobayashi,* and Tadashi Ariga*

Heterozygous gain-of-function (GOF) mutations of STAT1 are responsible for chronic mucocutaneous candidiasis disease (CMCD), one of the primary immunodeficiency diseases characterized by susceptibility to mucocutaneous Candida infection. To date, 30 aa changes have been reported: 21 in the coiled-coil domain and 9 in the DNA-binding domain. In this study, we report two novel STAT1 GOF mutations of p.K278E in coiled-coil domain and p.G384D in DNA-binding domain in Japanese CMCD patients. Ectopic expression of these STAT1 mutants in HeLa cells was associated with increased phosphorylation of the mutant and the endogenous wild-type STAT1 due to impaired dephosphorylation, indicating heterodimers of the mutants. Because IL-17A production was not significantly reduced at least in one of the patients following PMA plus ionomycin stimulation, we further studied Th17-associated cytokines IL-17A, IL-17F, and IL-22 in response to more physiologically relevant stimulations. IL-17A and IL-22 production from PBMCs rather than IL-17F was associated with the development of CMCD in these patients. Additionally, only the anti–IL-17F autoantibody in response to anti-CD3 plus anti-CD28 Abs stimulation. These results indicate impaired production of IL-17A and IL-22 rather than IL-17F was associated with the development of CMCD in these patients. Additionally, only the anti–IL-17F autoantibody was detected in sera from 11 of 17 patients with STAT1 GOF mutations, which may be useful as a marker for this disease.

The Journal of Immunology, 2014, 193: 000–000.

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Published October 6, 2014, doi:10.4049/jimmunol.1401467

The Journal of Immunology

C hronic mucocutaneous candidiasis (CMC) is characterized by susceptibility to Candida infection of the skin, nails, and mucosal membrane. CMC can occur as one of the various manifestations in patients with primary immunodeficiency diseases such as SCID, STAT3-deficient hyper-IgE syndrome (HIES), and autoimmune polyendocrine syndrome type 1 (APS1). Alternatively, CMC can be the only manifestation in patients with IL-17F deficiency, IL-17RA deficiency, or recently identified ACT1 deficiency (1, 2). This condition is described as isolated CMC or CMC disease (CMCD). Extensive analyses of CMCD patients resulting from impaired Th17 immunity have indicated the role of Th17 cells and Th17-associated cytokines in host defense against mucocutaneous Candida infection. In 2011, heterozygous STAT1 mutations were reported as a cause of CMCD (3, 4), and since then, 30 aa changes have been reported: 21 in the coiled-coil domain (CCD) and 9 in the DNA-binding domain (DBD) (see Fig. 1A) (3–13). Accumulated data indicated these mutations account for around half of CMCD patients and were associated with gain of STAT1 function due to impaired dephosphorylation of STAT1 (4, 13). The development of CMCD in this disorder could be also attributable to impaired Th17 immunity (3, 4, 13, 14), although the precise mechanisms have not been elucidated. In the present study, we describe two novel STAT1 mutations of p.K278E and p.G384D in CCD and DBD, respectively, in three CMC patients. These mutations were associated with gain of STAT1 function due to impaired dephosphorylation of STAT1, but the proportion of IL-17A* cells was not significantly reduced in one of the patients after PMA plus ionomycin (IOM) stimulation. Thus, we further studied Th17-associated cytokines IL-17A, IL-17F, and IL-22 in response to more physiologically relevant stimulations (15) and found impaired production of IL-17A and IL-22 but not IL-17F. We also studied autoantibodies against Th17-associated cytokines in 17 patients with STAT1 GOF mutations based on the facts that these patients manifest various autoimmune disorders (3, 4), and that patients with APS1 exhibiting various autoimmune disorders were shown to have neutral-
izing autoantibodies against various Th17-associated cytokines that could be associated with the development of CMC (16, 17). Patients with STAT1 GOF mutations were demonstrated to have anti–IL-17F autoantibody, although no neutralizing activity was observed.

Materials and Methods

Patients

Patient 1 is a 20-year-old woman born to nonconsanguineous healthy Japanese parents. Recurrent oral thrush developed since the age of 1 y. She also had recurrent herpetic zoster more than five times since the age of 4 y, and recurrent stomatitis about once every 2 mo that persisted around a week every time. Candida esophagitis developed at the age of 18 y. Impetigo con- tagiosa on the right thigh developed at the age of 19 y.

Patient 2 is a 35-year-old woman who had suffered from repetitive oral thrush and stomatitis since infancy. She had atopic dermatitis treated by anti- allergy drugs and topical corticosteroid. Bronchiectasis developed at the age of 18 y. Esophageal stenosis possibly caused by Candida esophagitis developed at the age of 19 y, which required balloon dilatation for swallowing foods. Iron-deficiency anemia had developed since her late 20s. She suffered from herpes zoster infection three times at the age of 11, 30, and 33 y. Patient 3 is a 36-year-old woman who is a son of patient 2. He had oral thrush and onychomycosis, and was diagnosed as having CMC at the age of 1 y. Herpes zoster infection developed at the age of 4 y.

Patients 1, 2, and 3 had normal proportion of lymphocytes and their subsets with normal levels of serum γ-globulins. These 3 patients had not presented with endocrine diseases or autoimmune diseases.

Patient 4 is a 14-year-old boy with a heterozygous GOF mutation of p.T273M affecting the DBD of STAT1 as described previously (13). We studied patient 4 as a control to study the phosphorylation state of STAT1 GOF.

Patient 5 is an 18-year-old woman with a heterozygous GOF mutation of p.R274Q affecting the CCD of STAT1 as described previously (13). We studied patient 5 as a control to analyze Th17-associated cytokine production.

Informed consent for genetic analysis was obtained from the patients as well as normal controls under a protocol approved by the Institutional Review Board of Hokkaido University Hospital.

DNA isolation, PCR, and sequence analysis of PCR products

These procedures were performed following the methods described elsewhere (18).

Generation of EBV-transformed cell lines

EBV-transformed cell lines (EBV-LCLs) were generated by in vitro transformation of human B cells with EBV (strain B95-8) as described elsewhere (18). Based on the results of STAT1 sequence analysis, EBV-LCLs from patients 1, 3, and 4 with the heterozygous mutations of p.K278E, p.G384D, and p.T385M were designated as K278E/wild-type (WT), G384D/WT, and T385M/WT, respectively. Two EBV-LCLs from healthy controls were designated as WT1 and WT2.

Measurement of IFN-γ-inducible protein 10 concentration in supernatant of EBV-LCLs using cytometric bead array

This procedure was performed following the methods described elsewhere (13).

Transient transfection of plasmids expressing STAT1–enhanced GFP WT or mutants into HeLa cells

To evaluate the phosphorylation state of the ectopically expressed STAT1 and the endogenous WT STAT1 separately, we prepared the constructs expressing STAT1–enhanced GFP (EGFP) fusion protein, which was made by inserting WT STAT1 cDNA into the pEFP-N1 vector (BD Bio- sciences, San Diego, CA) multicloning site. We generated STAT1 mutants with K278E, G384D, or T385M by mutagenesis (PrimeSTAR mutagenesis basal kit, TaKaRa Bio, Shiga, Japan) following the manufacturer’s protocol. HeLa cells were stripped by trypsin treatment 7 h before transfection and replaced at a density of 2.5 × 105 cells/ml in six-well plates. Plasmid DNA (5 μg/plate) carrying the WT or the various mutants or without STAT1 alleles was used for cell transfection with the TransFast LipoFectamine (Life Technologies, Carlsbad, CA). HeLa cells stimulated with recombinant human (rh)IFN-γ or rhIFN-α 3 h after transfection were harvested by trypsin. Transfection efficiencies were evaluated by FACS Calibur on the basis of the EGFP+ cells.

Studies of STAT1 phosphorylation state and staurosporine treatment of cells

A total of 1 × 105 cells/ml EBV-LCLs in RPMI 1640 with 10% FBS or transfected HeLa cells as described above were stimulated with 1:1000 diluted rhIFN-γ (Shionogi, Osaka, Japan) for 30 min, or 1500 U/ml rhIFN-α (BioSource International, Camarillo, CA) for 15 min in 5% CO2 at 37°C. For staurosporine treatment, these cells were then incubated with 1 μM staurosporine (Alomone Labs, Jerusalem, Israel), the tyrosine kinase in- hibitor, in 0.5% DMSO final concentration for 15 min successively. The cells were harvested, and nuclear extracts obtained as described previously (13) were subjected to immunoblot analysis.

Immunoblot analysis for the studies of STAT1 phosphorylation state

This procedure was performed basically following the methods described previously with minor modification (13). Briefly, the SNAP Liq. 2.0 protein detection system (EMD Millipore, Billerica, MA) was used for the de- tection of STAT1, p-STAT1, and lamin A based on the manufactures protocols. All of the primary Abs were used at 1:2500 dilution. HRP- conjugated anti-mouse IgG secondary Ab (GE Healthcare, Bucking- hamshire, U.K.) was used at 1:2500 dilution. The blots were then visualized by Luminata Forte Western HRP substrate (EMD Millipore).

Analysis of the production of IL-17A, IL-17F, IL-22, IFN-γ, and IL-4 from PBMCs and CD4+ cells

PBMCs were recovered by centrifuging blood samples on Ficoll gradients. CD4+ cells were positively selected by magnet sorting using CD4 microbeads (human) (Miltenyi Biotec, Auburn, CA) from PBMCs fol- lowing the manufacturer’s instructions. They were then adjusted to 1 × 107 cells/ml in RPMI 1640 containing 10% FBS. For stimulation with anti-CD3 plus anti-CD28 Abs (CD3/28), 1 × 106 cells/ml PBMCs were stimu- lated with 25 μl Dynabeads Human T-Activator CD3/28 (Life Technologies, Oslo, Norway) for 72 h. For Candida stimulation, PBMCs were stimulated with 100 μg/ml Candida Ag for skin test (Torii Pharmaceutical, Tokyo, Japan) for 72 h. CD4+ cells were stimulated with 25 μl Dynabeads Human T-Activator CD3/28 for 1 × 106 cells/ml for 72 h, and 400 ng/ml PMA and 10 μg/ml IOM for 6 h. The concentration of IL-17A, IL-17F, IFN-γ, and IL-4 in the supernatant was measured by cytokometric bead array (BD Biosciences) following the manufacturer’s instructions. IL-22 was measured with a human IL-22 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Data from triplicate independent experiments are reported as the means ± SD. The p values were calculated with a Mann–Whitney U test. The purity of CD4+ cells was examined with FACS Caltibur using anti-human PE-Cy5-conjugated anti-CD4 Ab (BioLegend, San Diego, CA).

Flow cytometric analysis of intracellular IL-17A expression in CD4+ cells

PBMCs at a density of 1 × 106 cells/ml were stimulated with 500 ng/ml PMA plus 5 μg/ml IOM for 6 h. We added of 10 μg/ml brefeldin A for the last 2 h. Harvested PBMCs were washed with TBST and stained with allophycocyanin-conjugated anti-human CD4 Ab (BioLegend) for 20 min. Cells were washed three times with PBS and fixed and permeabilized with a Foxp3 staining buffer set (eBioscience, San Diego, CA) for 20 min at 4°C. Cells were then washed three times and incubated for 30 min with PE-conjugated anti-human IL-17A (BioLegend). Cells were washed three times and analyzed with a BD LSRFortessa.

Detection of anti-cytokine autoantibodies in sera from patients by immunoblot analysis

Five hundred nanograms rhIL-17A, IL-17F, IL-17, IL-22, IL-23, IL-1( B i o L e g e n d ) were recovered from centrifuging blood samples on Ficoll gradients. CD4+ cells were positively selected by magnet sorting using CD3 microbeads (human) (Miltenyi Biotec, Auburn, CA) from PBMCs fol- lowing the manufacturer’s instructions. They were then adjusted to 1 × 107 cells/ml in RPMI 1640 containing 10% FBS. For stimulation with anti-CD3 plus anti-CD28 Abs (CD3/28), 1 × 106 cells/ml PBMCs were stimu- lated with 25 μl Dynabeads Human T-Activator CD3/28 (Life Technologies, Oslo, Norway) for 72 h. For Candida stimulation, PBMCs were stimulated with 100 μg/ml Candida Ag for skin test (Torii Pharmaceutical, Tokyo, Japan) for 72 h. CD4+ cells were stimulated with 25 μl Dynabeads Human T-Activator CD3/28 for 1 × 106 cells/ml for 72 h, and 400 ng/ml PMA and 10 μg/ml IOM for 6 h. The concentration of IL-17A, IL-17F, IFN-γ, and IL-4 in the supernatant was measured by cytokometric bead array (BD Biosciences) following the manufacturer’s instructions. IL-22 was measured with a human IL-22 Quantikine ELISA kit (R&D Systems, Minneapolis, MN). Data from triplicate independent experiments are reported as the means ± SD. The p values were calculated with a Mann–Whitney U test. The purity of CD4+ cells was examined with FACS Calibur using anti-human PE-Cy5-conjugated anti-CD4 Ab (BioLegend, San Diego, CA).

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Results
Two novel amino acid substitutions in STAT1

Direct sequence analysis demonstrated that patient 1 had a heterozygous base change of c.832A→G, p.K278E (K278E) in CCD, and the parents of patient 1 had WT sequence in STAT1. Both patients 2 and 3 had the heterozygous base change of c.1150G→A, p.G384D (G384D) in DBD of STAT1 (Fig. 1A, 1B). These base changes have not been reported either as disease-causing mutations or as single nucleotide polymorphisms in the National Center for Biotechnology Information database, the Ensembl database, the Single Nucleotide Polymorphism Database, and the Human Genetic Variation Database, which have information on 1208 Japanese single nucleotide polymorphisms, or in our 100 controls without CMC (data not shown). Both K278E and G384D were evolutionarily conserved (Fig. 1C). K278E was predicted as tolerated by the sort intolerant from tolerant algorithm, and it was predicted as benign with a score of 0.011 (sensitivity, 0.96; specificity, 0.78) by the polymorphism phenotype-2 algorithm. G384D was predicted as tolerated by the sort intolerant from tolerant algorithm but was predicted as probably damaging by the polymorphism phenotype-2 algorithm with a score of 1.000 (sensitivity, 0.00; specificity, 1.00).

K278E and G384D were associated with STAT1 GOF and increased STAT1 phosphorylation after IFN-γ and IFN-α stimulation

Heterozygous STAT1 GOF mutations were shown to be the genetic causes of autosomal dominant or sporadic CMC. The reported mutations were associated with increased STAT1 phosphorylation due to impaired dephosphorylation. First, to study whether the amino acid substitutions of K278E and G384D in STAT1 also lead to STAT1 GOF, the production of IFN-γ-inducible protein 10 (IP10), the downstream target of STAT1, was analyzed. IP10 production was significantly higher in K278E/WT and G384D/WT EBV-LCLs compared with healthy controls following IFN-γ stimulation (Fig. 2A). We then studied the STAT1 phosphorylation state in EBV-LCLs from the patients and controls. Expression of p-STAT1 following IFN-γ or IFN-α stimulation was higher in G384D/WT, K278E/WT, and T385M/WT than in WT1 EBV-LCLs (Fig. 2B, 2C). Ectopic expression of STAT1 mutants was associated with increased phosphorylation of the STAT1 mutants in HeLa cells (Fig. 2D). These results indicate that K278E and G384D are GOF mutations responsible for CMCD, and the mechanism of GOF is STAT1 hyperphosphorylation following the IFNs stimulations.

FIGURE 1. Two heterozygous base changes of STAT1 leading to amino acid substitutions were identified in three CMCD patients. (A) The reported heterozygous STAT1 mutations responsible for CMCD are shown. Mutations identified in this study are shown in red. (B) Direct sequence analysis of STAT1 exon 10 in patient 1 (P1) and exon 14 in patients 2 (P2) and 3 (P3). Forward sequences are shown. (C) Comparison of the amino acid sequences of STAT1 in different species. The red rectangles indicate the amino acids corresponding to p.K278 or p.G384 in humans, respectively.
Increased phosphorylation of the STAT1 mutants was due to impaired dephosphorylation

The mechanisms underlying increased phosphorylation of the STAT1 mutants were further investigated with the tyrosine kinase inhibitor staurosporine. Dephosphorylation of the ectopically expressed STAT1-EGFP K278E or G384D mutants was impaired in HeLa cells following IFN-γ or IFN-α stimulation, compared with that of STAT1-EGFP WT (Fig. 3). Therefore, the mechanisms underlying increased STAT1 phosphorylation of K278E and G384D mutants involve impaired dephosphorylation of STAT1.

Increased phosphorylation and impaired dephosphorylation of the endogenous WT STAT1 in addition to the ectopically expressed mutants in HeLa cells

The endogenous WT STAT1 was also demonstrated to have increased STAT1 phosphorylation following IFN-γ stimulation when

FIGURE 2.  G384D and K278E were associated with GOF and increased phosphorylation of STAT1 in response to IFN-γ or IFN-α stimulation.  (A) IP10 production from EBV-LCLs stimulated with or without IFN-γ for 6 h.  (B and C) p-STAT1 and STAT1 expression in EBV-LCLs following IFN-γ stimulation for 30 min (B), and IFN-α stimulation for 15 min (C).  (D) p-STAT1 and STAT1 expression in transfected HeLa cells following IFN-γ stimulation for 30 min. Transfection efficiencies were analyzed on the basis of GFP + cells and are described under each lane.

FIGURE 3.  G384D and K278E were associated with increased phosphorylation of STAT1 due to impaired dephosphorylation.  The p-STAT1 expression is shown in transfected HeLa cells first stimulated with IFN-γ for 30 min (A) or IFN-α for 15 min (B) followed by incubation with 1 μM staurosporine for 15 min. Transfection efficiencies are described under each lane.
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-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-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-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. Production of 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 STAT1 GOF patients 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](http://www.jimmunol.org/)

Patients 1 and 3 had reduced CD4\(^+\)IL-17A\(^+\) cells in response to PMA plus IOM stimulation, whereas patient 2 had a normal proportion of CD4\(^+\)IL-17A\(^+\) cells compared with controls.
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
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**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Positive Autoantibodies</th>
<th>Negative Autoantibodies</th>
<th>oIL17F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ANA 160×, microsome 100×, TRAb 1.0 (&lt; 0.9 IU/l)</td>
<td>TgAb</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>dsDNA 10 (&lt;10 IU/ml)</td>
<td>ANA, dsDNA, TRAb, TPO, TgAb, GAD, IAA, direct Coombs test</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>TRAb (blocking rate 16% [&lt;15%])</td>
<td>ANA, TRAb, TPO, TgAb, GAD, IAA, direct Coombs test</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>TgAb 55.33 (&lt;40 IU/ml)</td>
<td>ANA, dsDNA, TRAb, TPO, GAD, IAA, IA-2, CCP, RF, c-ANCA, p-ANCA, AMA, αPL</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>ANA 160×</td>
<td>TPO, TgAb</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Direct and indirect Coombs test</td>
<td>ANA, TRAb</td>
<td>+</td>
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<tr>
<td>7</td>
<td>ANA 40×</td>
<td>TPO, TgAb</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>ANA 40×</td>
<td>dsDNA, TPO, TgAb, GAD, IAA, IA-2</td>
<td>+</td>
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<tr>
<td>9</td>
<td>ANA 40×</td>
<td>TRAb</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>ANA 80×</td>
<td>ANA, GAD, IAA, IA-2</td>
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<td>15</td>
<td>ANA, TPO, TRAb, ASMA</td>
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<td>17</td>
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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; oIL17F, 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 CMCD.

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.

Acknowledgments
We thank the patients and their families for the collaboration in this study.

Disclosures
The authors have no financial conflicts of interest.

References