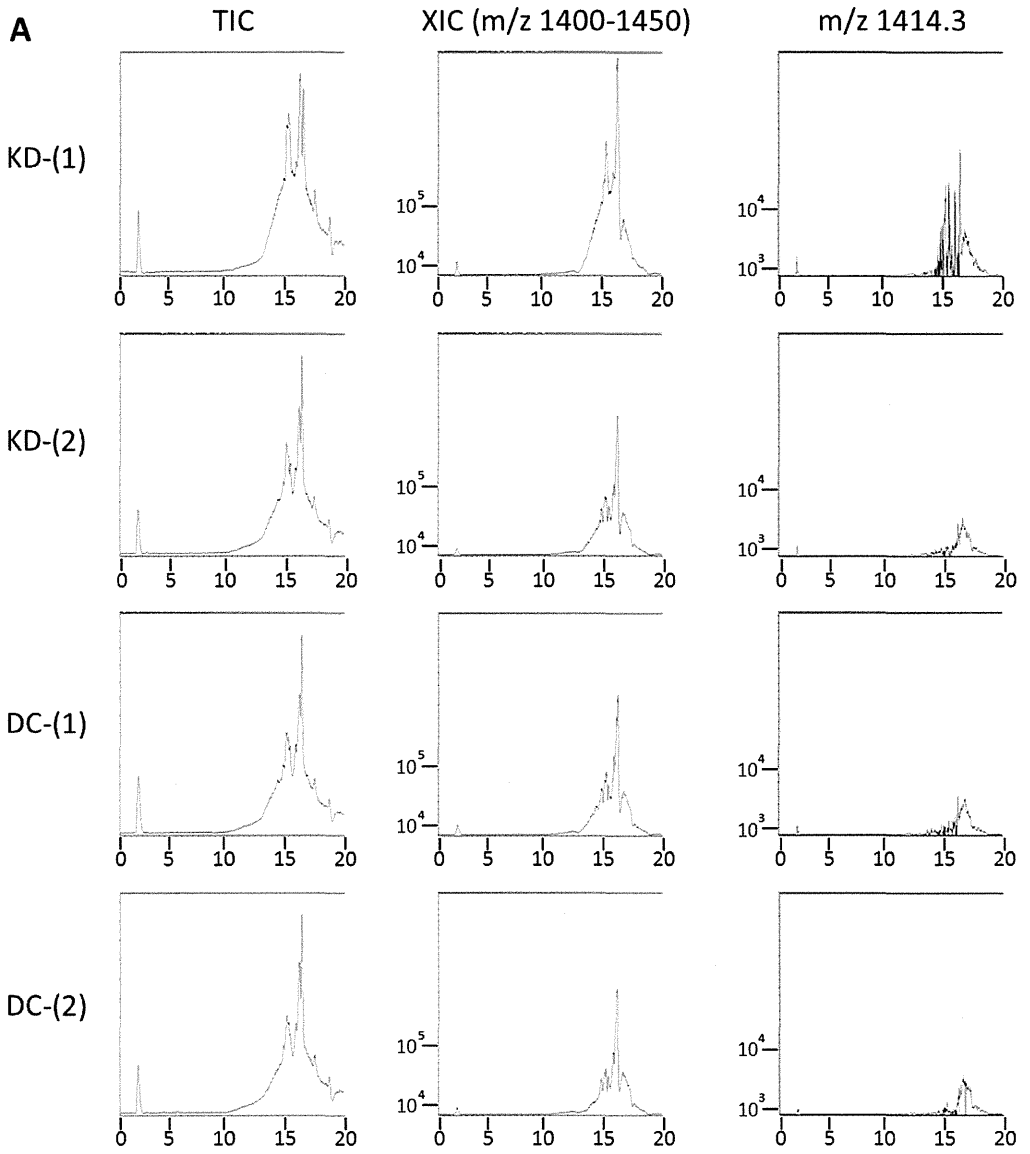

B

Affinity column	Binding of a KD specific molecule
Human polyclonal IgG column	+
Mouse monoclonal IgG column	+
Rabbit monoclonal IgG column	+
Human IgG $F(ab')_2$ column	+
Human Fc-column	±
Control column	−

Figure 5. LC-MS chromatograms of IgG sepharose-binding molecules. A. Representative LC-MS chromatograms of a IgG sepharose-binding molecule (m/z 1414.3) are shown in a KD patient and a DC control. TIC: Total ion current chromatograms, XIC: Extracted-ion chromatograms at m/z 1400–1500, and extracted-ion chromatograms at m/z 1414.3. (1) Human polyclonal IgG-conjugated sepharose 6 Fast (2) Inactivated CNBr Sepharose 4B control column. B. Binding of a KD-specific molecule to various affinity columns: Columns used are described in ONLINE METHODS. +: The binding quantities of a KD-specific molecule analyzed by LC-MS were equal or larger than those to human polyclonal IgG column, ±: smaller than 20% of those to human polyclonal IgG column, -: no binding. We performed the experiments 3 times.
doi:10.1371/journal.pone.0113054.g005

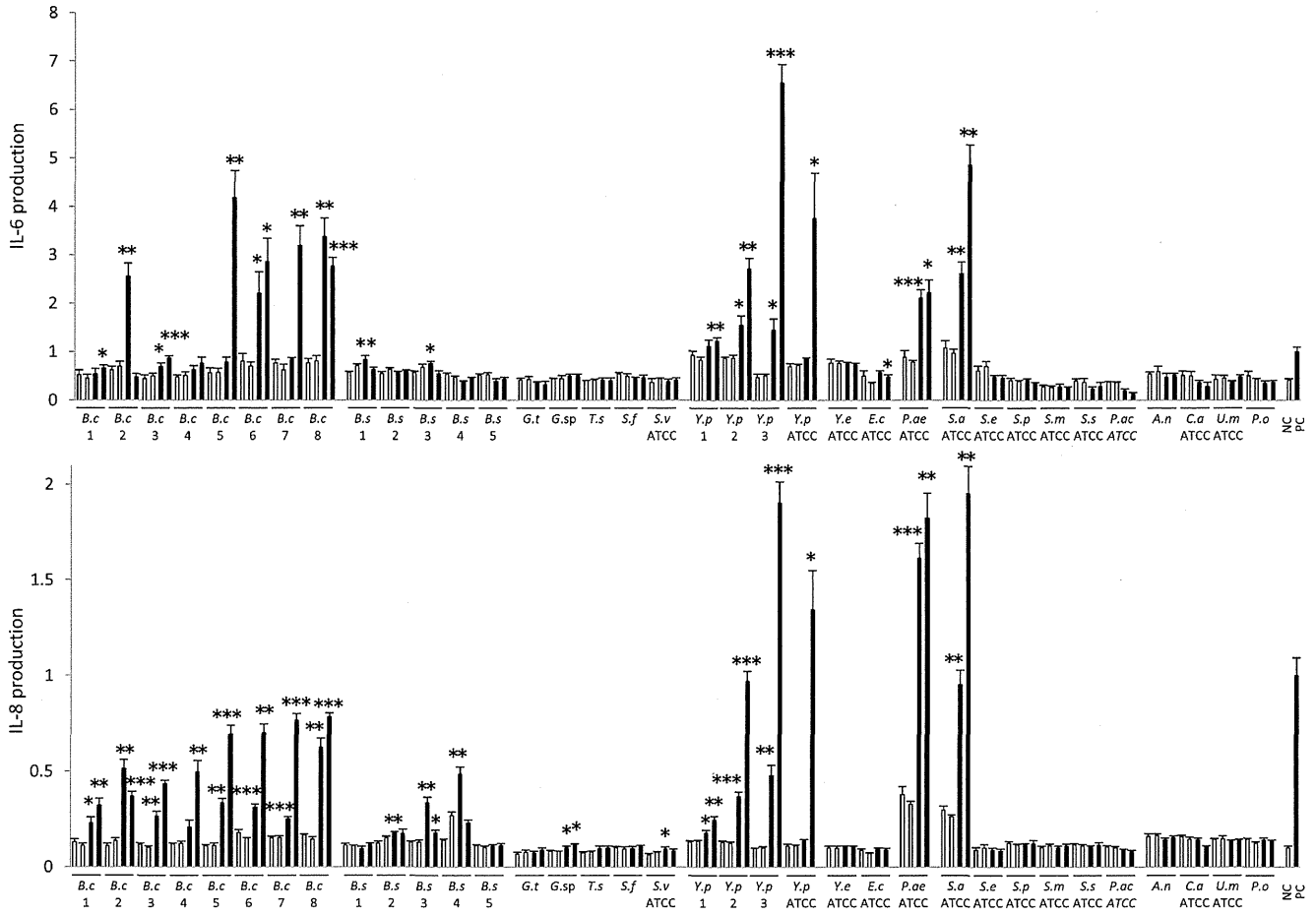

Figure 6. Activation of HCAECs by biofilm lipid extracts from various microbes. The production of IL-6 and IL-8 by HCAECs was measured after 24-h culture in the presence or absence of a microbial stimulant. Each microbe was cultured in the presence or absence of biofilm-forming glass slides with or without butter. As a microbial stimulant, an extract from a culture supernatant (□) or a biofilm (■) of a microbe cultured in the presence (right column) or absence (left column) of butter was used. Medium alone, ethyl acetate alone or ethyl acetate extract from glass slides cultured in the absence of a microbe was used as a negative control (NC). FK 565 (10 μg/mL) was used as a positive control (PC). *B.c: Bacillus cereus, B.s: Bacillus subtilis, G.t: Gordonia terrae, T.s: Terribacillus saccharophilus, S.f: Streptomyces flavogriseus, S.v: Streptomyces violaceus, Y.p: Yersinia pseudotuberculosis, Y.e: Yersinia enterocolitica, E.c: Escherichia coli, P.ae: Pseudomonas aeruginosa, S.a: Staphylococcus aureus, S.e: Staphylococcus epidermidis, S.p: Streptococcus pyogenes, S.m: Streptococcus mitis, S.s: Streptococcus sanguinis, P.ac: Propionibacterium acnes, A.n: Aspergillus niger, C.a: Candida albicans, U.m: Ustilago maydis, P.o: Penicillium oxalicum*. Numbers under bacteria indicate those of KD patients. Data are expressed as the fold change induction of IL-8 or IL-6 compared to the PC levels. We performed the experiments 3 times. Biofilms were compared with supernatant control considering presence or absence of butter. *$P<0.01$, **$P<0.001$ and ***$P<0.0001$ (Welch's *t*-test).
doi:10.1371/journal.pone.0113054.g006

fractionated crude biofilm extracts were too toxic to replicate the KD phenotype in mice.

We have shown that serum KD-specific molecules were diverse but mostly derived from biofilms and possessed molecular structures common to MAMPs. The present study suggests a possibility that KD-specific MAMPs induce vascular inflammation, leading to the development of KD. Further study is on the way as a nation-wide project to investigate a pathogenic link between KD development and biofilm-derived MAMPs.

Conclusion

Extensive analysis by LC-MS/MS revealed that serum KD-specific molecules possessed molecular structures common to MAMPs from *Bacillus cereus, Bacillus subtilis, Yersinia pseudotuberculosis and Staphylococcus aureus*. These molecules were mostly derived from biofilms formed *in vivo* (teeth, tongue, nasal cavity, or stool). This report might offer novel insight into the diagnosis and management of KD as well as its pathogenesis.

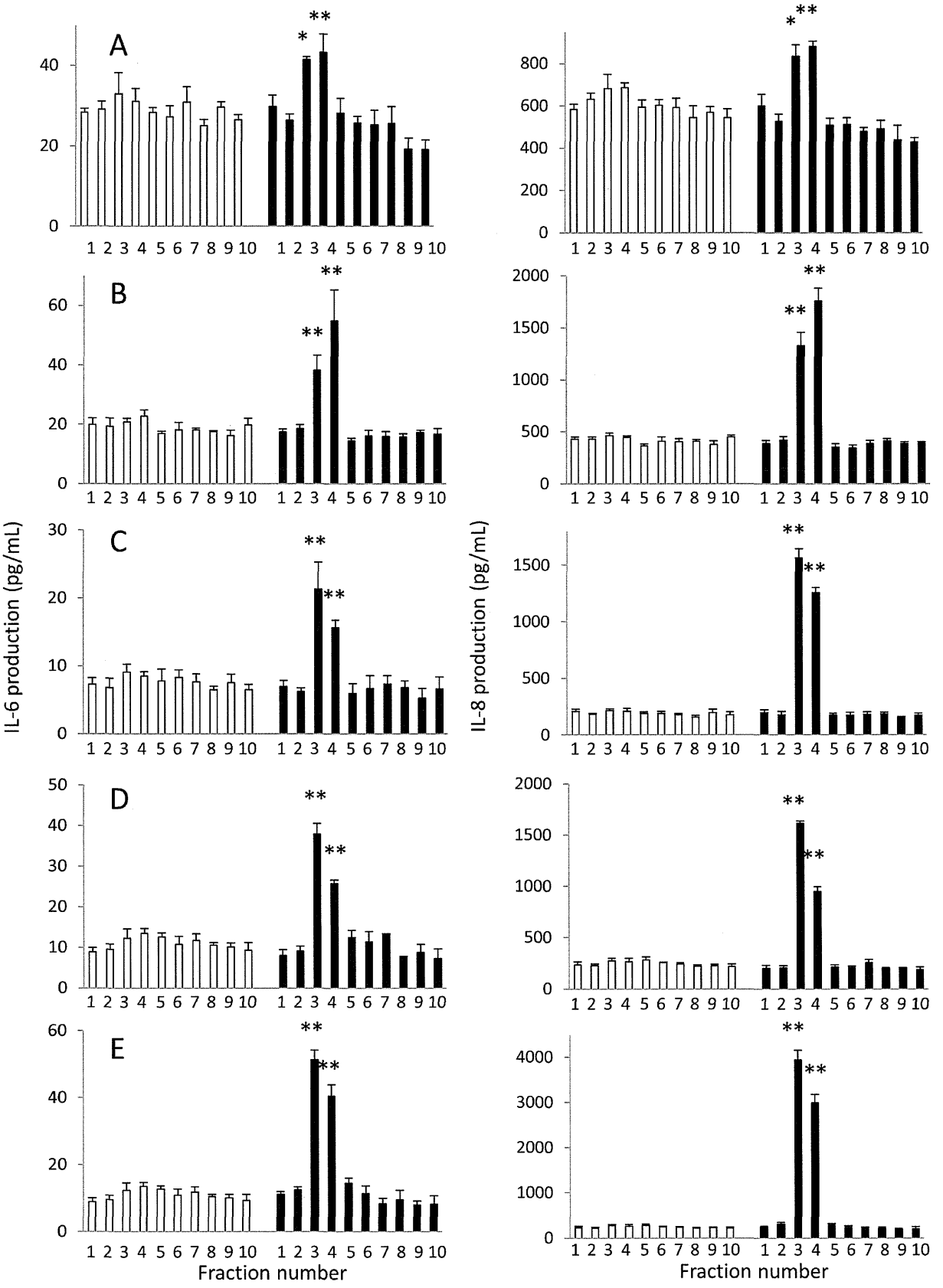

Figure 7. Fractionation of HCAEC-activating biofilm lipid extracts by HPLC. Each biofilm lipid extract from *Y. pseudotuberculosis* (A), *B. cereus* (B), *B. subtilis* (C), *S. aureus* (D) or *P. aeruginosa* (E) was separated into 10 fractions by HPLC and assayed for the stimulatory activity of HCAECs (■). Fractions 3 and 4 from each biofilm lipid extract induced high cytokine production by HCAECs. Ethyl acetate lipid extracts from glass slides in the

absence of microbes served as negative controls (□). Results are representative of 3 independent experiments. The stimulatory effects of fractionated biofilm samples were compared with those of corresponding controls. *$P<0.05$, **$P<0.01$ (Welch's *t*-test).
doi:10.1371/journal.pone.0113054.g007

Supporting Information

File S1 Supporting information. Text S1, Supporting Materials and Methods. Figure S1, LC-MS chromatograms and the detection rates and time course of 5 KD-specific molecules. Figure S2, Effects of biofilm formation, shaking time and various oils on the production of a KD-specific MAMP by LC-MS analysis. Figure S3, LC-MS and MS/MS analyses of 3 KD-specific molecules with IgG sepharose-binding activity. Table S1, Detection rates of spore-forming and pathogenic microbes in the oral cavity and upper respiratory tract of KD patients. Table S2, Presence of MAMPs in various microbes similar to serum KD-specific molecules. Table S3, Common MAMPs between the *in vivo* biofilms and sera in respective KD patients at the 2nd study. Table S4, Common MAMPs between the *in vivo* biofilms and sera, and microbes detected in respective KD patients at the 3rd study. Table S5, Sequences of oligonucleotide primers used for the amplification microbial genes.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TH. Performed the experiments: TK YN KM SK HN TT. Analyzed the data: TK YN KM HN TM TH. Contributed reagents/materials/analysis tools: TK YN KY YM KO KW. Wrote the paper: TK YN TH. Scientific input and contributed to discussion: MS YS HT.

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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 wild-type and mutant STAT1 cause impaired dephosphorylation, as did homodimers 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 and CD4$^+$ cells was significantly reduced in four patients with *STAT1* GOF mutations, including the previously reported R274Q in response to anti-CD3 plus anti-CD28 Abs or *Candida* stimulations. In contrast, IL-17F production was comparable to healthy controls 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: 4880–4887.**

Chronic 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-

*Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo 060-8638, Japan; †Department of Human Genetics, National Center for Child Health and Development, Tokyo 157-8535, Japan; ‡Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Tokyo 113-8519, Japan; §Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan; and ¶Department of Dentistry for Children and Disabled Persons, Hokkaido University Graduate School of Dental Medicine, Sapporo 060-8586, Japan

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Address correspondence and reprint requests to Dr. Masafumi Yamada, Department of Pediatrics, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan. E-mail address: yamadam@med.hokudai.ac.jp

Abbreviations used in this article: ANA, anti-nuclear Ab; APS1, autoimmune polyendocrine syndrome type 1; CCD, coiled-coil domain; CD3/28, anti-CD3 plus anti-CD28 Abs; CMC, chronic mucocutaneous candidiasis; CMCD, chronic mucocutaneous candidiasis disease; DBD, DNA-binding domain; EGFP, enhanced GFP; GOF, gain-of-function; HIES, hyper-IgE syndrome; ILC, innate lymphoid cell; IOM, ionomycin; IP10, IFN-γ–inducible protein 10; LCL, lymphoid cell line; rh, recombinant human; WT, wild-type.

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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-y-old woman born to nonconsanguineous healthy Japanese parents. Recurrent oral thrush developed since the age of 1 y. She also had recurrent herpes 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 contagiosa on the right thigh developed at the age of 19 y.

Patient 2 is a 35-y-old woman who had suffered from repetitive oral thrush and stomatitis since infancy. She had atopic dermatitis treated by antiallergy 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 5-y-old boy 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-y-old boy with a heterozygous GOF mutation of p.T385M 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-y-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-lymphoid cell lines [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 pEGFP-N1 vector (BD Biosciences, Franklin Lakes, NJ) 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 2.5×10^5 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 TransIT-LT1 reagent (Mirus Bio, Madison, WI). HeLa cells stimulated with recombinant human (rh)IFN-γ or rhIFN-α 36 h after transfection were harvested by trypsin. Transfection efficiencies were evaluated by FACSCalibur on the basis of the $EGFP^+$ cells.

Studies of STAT1 phosphorylation state and staurosporine treatment of cells

A total of 1×10^6 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% CO_2 at 37°C. For staurosporine treatment, these cells were then incubated with 1 μM staurosporine (Alomone Labs, Jerusalem, Israel), the tyrosine kinase inhibitor, 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 i.d. 2.0 protein detection system (EMD Millipore, Billerica, MA) was used for the detection 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, Buckinghamshire, 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 following the manufacturer's instructions. They were then adjusted to 1×10^6 cells/ml in RPMI 1640 containing 10% FBS. For stimulation with anti-CD3 plus anti-CD28 Abs (CD3/28), 1×10^6 cells/ml PBMCs were stimulated 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 1×10^6 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 with cytometric 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 FACSCalibur 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 1×10^6 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-22, IL-23, IL-1β, IL-6 (R&D Systems), IFN-α (BioSource International), and TGF-β1 (BioLegend) were separated by 15% polyacrylamide gels and transferred to Immobilon-P transfer membranes. The membranes were blocked by TBST with 5% nonfat skim milk for 1 h. They were then reacted with the sera at 1:1500 dilutions overnight at 4°C. The sera involved 17 STAT1 GOF CMC patients, 2 APS1 patients, 2 STAT3-deficient HIES patients, and 21 healthy controls. After washing four times with TBST, the membranes were incubated with HRP-conjugated goat anti-human IgG or IgA (Invitrogen, Frederick, MD) at 1:150,000 or 1:40,000 dilutions, respectively, in TBST with 5% non-fat skim milk for 1 h at 4°C. After washing four times, the blots were then visualized by ECL Select Western blotting detection reagent (GE Healthcare). To titrate anti–IL-17F autoantibody, we performed immunoblot analysis of sera from the patients serially diluted from 1:640 to 1:81,920.

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.

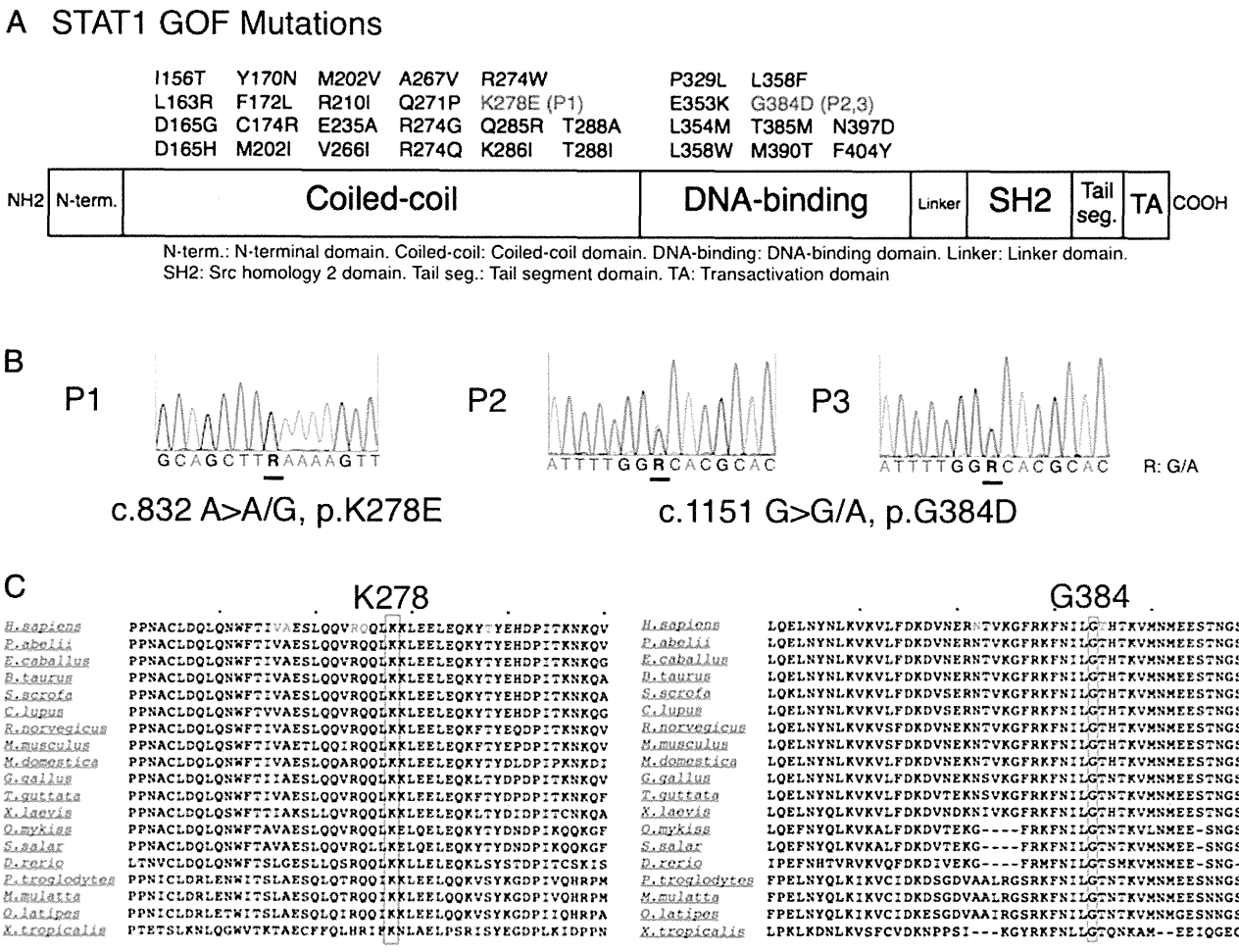

FIGURE 1. Two heterozygous base changes of *STAT1* leading to amino acid substitutions were identified in three CMCD patients. (**A**) The reported heterozygous *STAT1* mutations responsible for CMCD are shown. Mutations identified in this study are shown in red. (**B**) Direct sequence analysis of *STAT1* 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.

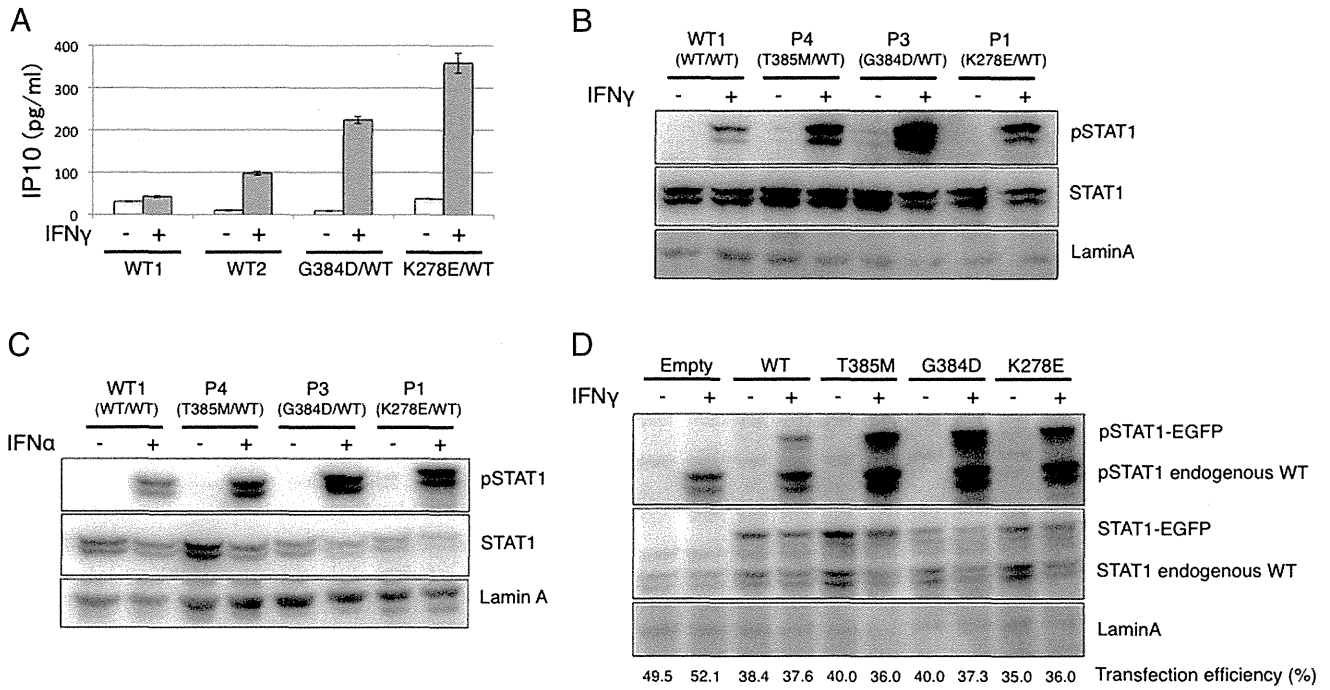

FIGURE 2. G384D and K278E were associated with GOF and increased phosphorylation of STAT1 in response to IFN-γ or IFN-α stimulation. (**A**) IP10 production from EBV-LCLs stimulated with or without IFN-γ for 6 h. (**B** and **C**) p-STAT1 and STAT1 expression in EBV-LCLs following IFN-γ stimulation for 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.

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

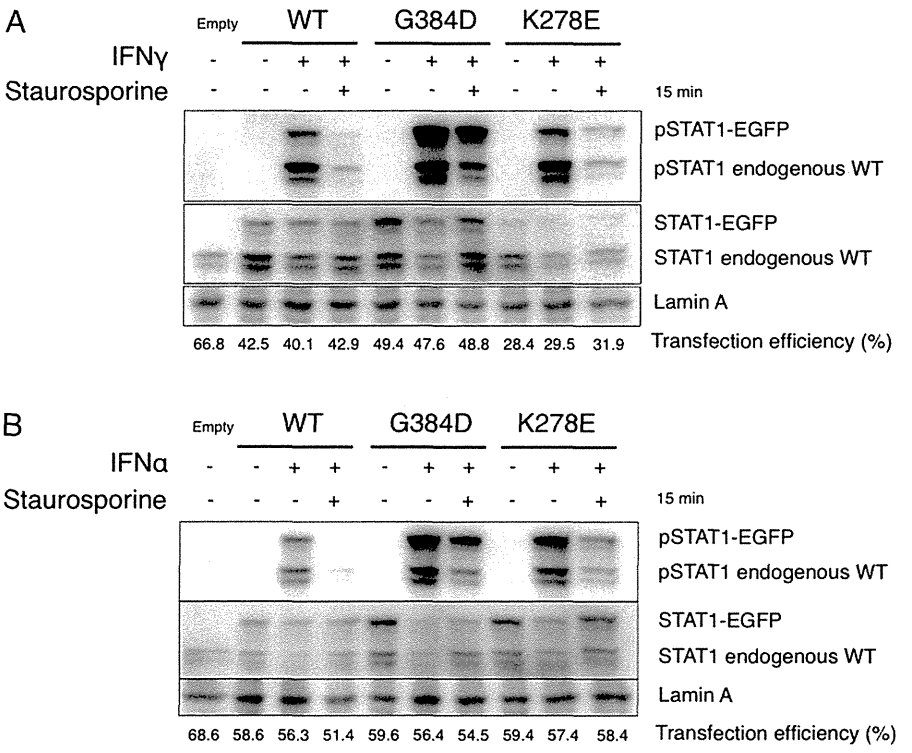

FIGURE 3. G384D and K278E were associated with increased phosphorylation of STAT1 due to impaired dephosphorylation. The p-STAT1 expression is shown in transfected HeLa cells first stimulated with IFN-γ for 30 min (**A**) or IFN-α for 15 min (**B**) followed by incubation with 1 μM staurosporine for 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 $CD4^+IL\text{-}17A^+$ 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

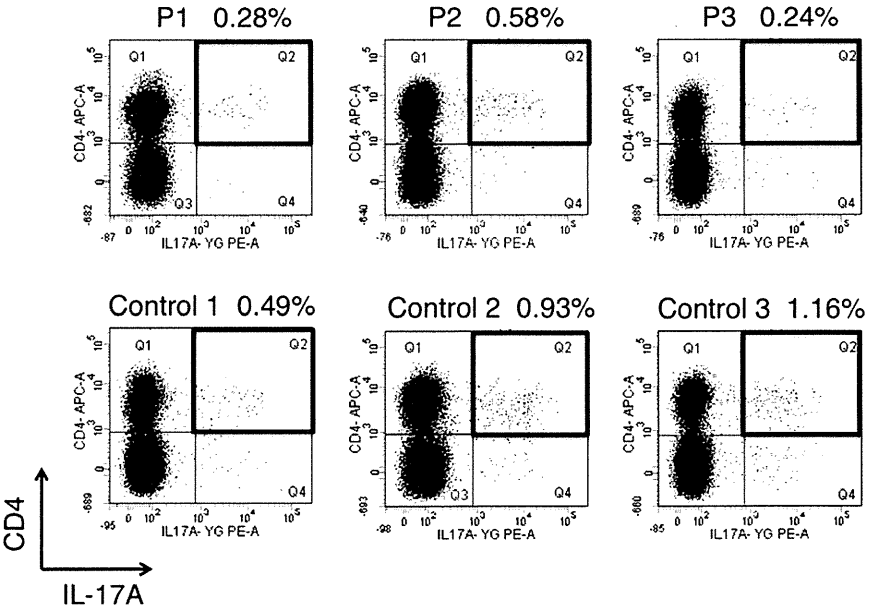

FIGURE 4. Patients 1 and 3 had reduced $CD4^+IL17A^+$ cells in response to PMA plus IOM stimulation, whereas patient 2 had a normal proportion of $CD4^+IL\text{-}17A^+$ cells compared with controls.

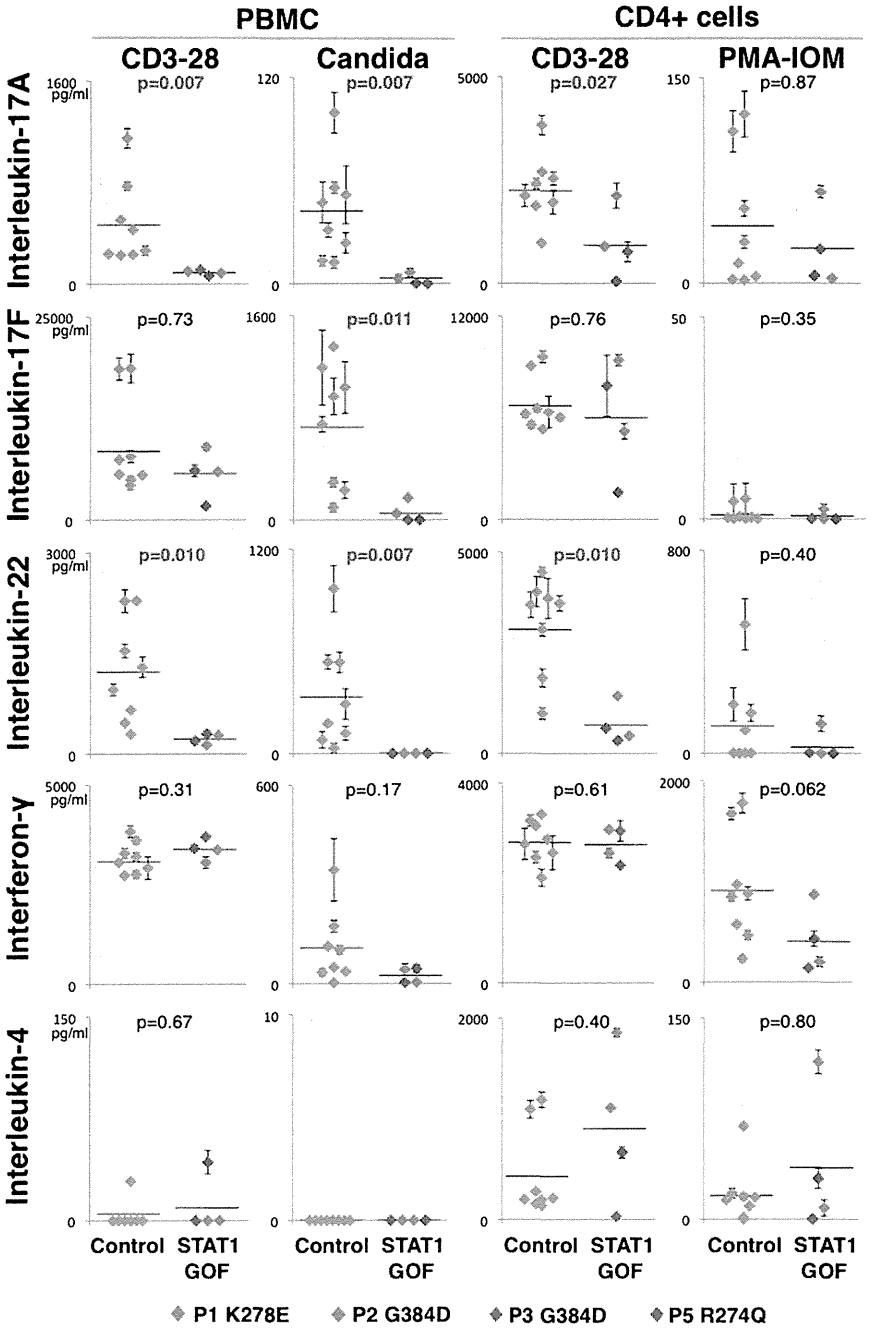

FIGURE 5. IL-17A and IL-22 production from PBMCs and CD4$^+$ cells was significantly reduced in four patients with STAT1 GOF in response to CD3/28 or *Candida* stimulations, whereas IL-17F production was comparable to healthy controls in response to CD3/28 stimulation. Productions of IL-17A, IL-17F, 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*

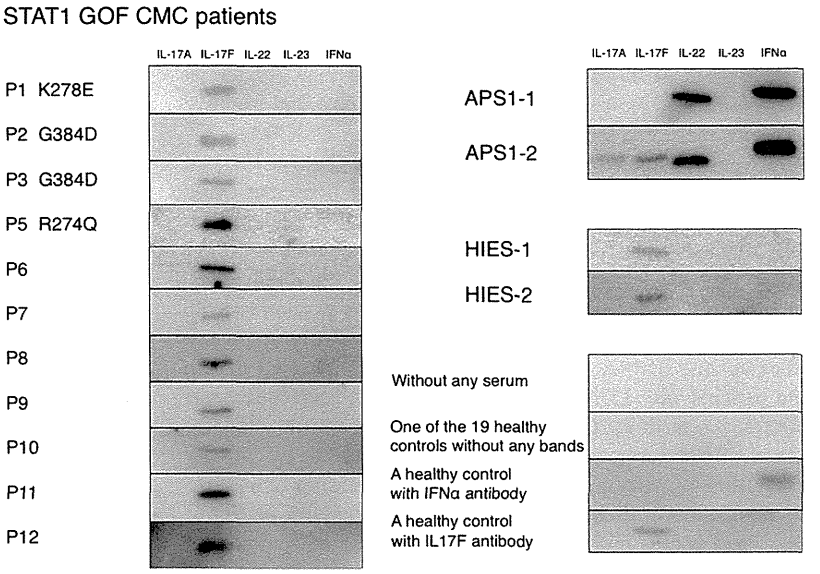

FIGURE 6. Immunoblot analysis showed the exclusive presence of anti–IL-17F autoantibody in the 11 of 17 STAT1 GOF patients' sera (64.7%). We confirmed the results by more than two independent experiments.

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.

Acknowledgments

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

Disclosures

The authors have no financial conflicts of interest.

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ORIGINAL RESEARCH

Efficacy and Safety of IgPro20, a Subcutaneous Immunoglobulin, in Japanese Patients with Primary Immunodeficiency Diseases

Hirokazu Kanegane · Kohsuke Imai · Masafumi Yamada · Hidetoshi Takada · Tadashi Ariga · Martin Bexon · Mikhail Rojavin · Wilson Hu · Midori Kobayashi · John-Philip Lawo · Shigeaki Nonoyama · Toshiro Hara · Toshio Miyawaki

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Abstract
Purpose Intravenous (IVIG) and subcutaneous (SCIG) immunoglobulin infusions are widely used for the treatment of patients with primary immunodeficiency (PID) worldwide. This prospective, multicenter, open-label, single-arm Phase III study evaluated the efficacy, tolerability, and safety of IgPro20 (Hizentra®; L-proline–stabilized 20 % human SCIG) in adult and pediatric Japanese patients with PID.
Methods Patients received three IVIG infusions at 3–4-week intervals followed by a dose-equivalent switch to weekly SCIG infusions. A 12-week wash-in/wash-out period was followed by a 12-week SCIG efficacy period. The primary efficacy endpoint was the comparison of total serum IgG trough levels during the IVIG and SCIG efficacy periods by calculating the geometric mean ratio (GMR).
Results The GMR of IgG trough levels on SCIG versus IVIG was 1.09 (2-sided 90 % confidence interval: 1.06–1.13). No serious bacterial infections were reported. Eleven patients (52.4 %) had infectious episodes with an overall rate of 2.98 infections/patient/year; 7 patients (33.3 %) missed school/work/daycare due to infection (3.48 days/patient/year). Sixteen patients (76.2 %) were treated with antibiotics for an adverse event (AE; 47.6 %) or prophylaxis (23.8 %), resulting in 167.42 days/patient/year of antibiotic use. During SCIG treatment, 24 patients (96.0 %) had 269 AEs (0.461 AEs per/infusion) including local reactions as the most common AE (20 patients, 80.0 %). Local tolerability of IgPro20 was assessed as "very good" or "good" after 85.4 % of SCIG infusions. One patient (4.0 %) experienced a serious AE of moderate severity (bacterial infection) that was considered unrelated to study medication.
Conclusion IgPro20 was effective and well tolerated in Japanese patients with PID.

Keywords Primary immunodeficiency · PID · primary antibody deficiency · SCIG · Hizentra® · IgPro20 · Japan

H. Kanegane
Department of Pediatrics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

K. Imai
Department of Community Pediatrics, Perinatal and Maternal Medicine, Tokyo Medical and Dental University, Tokyo, Japan

M. Yamada · T. Ariga
Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

H. Takada · T. Hara
Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

M. Bexon
CSL Behring AG, Berne, Switzerland

M. Rojavin
CSL Behring LLC, King of Prussia, PA, USA

W. Hu
CSL Limited, Research and Development, Melbourne, Victoria, Australia

M. Kobayashi
CSL Behring KK, Tokyo, Japan

J.-P. Lawo
CSL Behring GmbH, Marburg, Germany

S. Nonoyama
Department of Pediatrics, National Defense Medical College, Saitama, Japan

T. Miyawaki (✉)
Toyama City Hospital, Toyama, Japan
e-mail: toshio65@tch.toyama.toyama.jp

Introduction

Primary immunodeficiency (PID) includes a range of genetic disorders that are characterized by an intrinsic defect in the immune system (B- and T-cell defects, phagocytic disorders, and complement deficiencies) [1]. PID results in the patient's predisposition to recurrent multiple infections despite intensive treatment with antibiotics [2, 3].

Prevalence of PID in Japan was determined in a recent nationwide survey (N=1,240) to be 2.3 PID patients per 100,000 inhabitants, with minimal variations across the country regions [4]. These results are comparable with the prevalence of PID in Taiwan (0.77–2.17) and Singapore (2.7), but are lower than those in European countries (e.g., 4.4 in France), the Middle East (11.98), and the US (83.3) [4, 5].

Most patients with PID have a primary antibody deficiency (PAD) and require immunoglobulin G (IgG) replacement therapy with regular administration of IgG to prevent infection and maintain quality of life [3]. Intravenous immunoglobulin (IVIG) infusions every 3–4 weeks are the current standard practice in Japan. However, the likelihood of developing severe and/or systemic adverse reactions and the difficulties in obtaining venous access, particularly in children, have prompted the development of alternative modes of IgG delivery. Consequently, IgG administration by the subcutaneous route (SCIG) has been developed and is now available in Europe and the United States [6, 7]. IVIG and SCIG infusions are the current treatment modalities of choice for patients with PAD worldwide [8, 9].

Patients and their doctors select SCIG for better flexibility and freedom from long visits to clinics for IVIG infusions. SCIG does not require venous access, which is particularly welcome in children. Additional medical benefits of SCIG compared with IVIG include lower rates of systemic adverse events (AEs) and more even serum IgG levels between the infusions [6, 7, 10]. The latter ensures better protection against infections over the full period between the infusions and helps to alleviate or completely avoid the known "wear-off" effects, such as decrease of an overall well-being and higher probability of infection development during the last pre-infusion week [6, 11].

IgPro20 (Hizentra®; L-proline–stabilized 20 % human SCIG) is the first and only 20 % SCIG preparation approved in the US (2010) and Europe (2011) for treatment of PID. It has been found effective and well tolerated in two Phase III studies in both adult and pediatric patients with PID [6, 7, 12–14].

This is the first prospective, multicenter, open-label, single-arm study of SCIG in the Japanese population. Here we report the results of efficacy, tolerability, and safety evaluation of SCIG therapy in Japanese patients with PID after a dose-equivalent switch from IVIG.

Methods

Patients, Inclusion and Exclusion Criteria

Male and female outpatients 75 years of age or younger with PID requiring IgG replacement therapy were eligible for the study. The PID diagnosis was based on the diagnostic criteria defined by the Pan-American Group for Immunodeficiency and the European Society for Immunodeficiencies [15]. Only patients who had received at least three doses of IVIG at regular 3- or 4-week intervals at a stable dose (variations of ±10 % were allowed) prior to enrollment were eligible. For inclusion in the SCIG period, the patient's IgG trough levels between screening (or first mandatory IVIG infusion) and third mandatory IVIG infusion should have remained above or equal to 4 g/L, with at least one measurement above or equal to 5 g/L.

Patients who developed a serious bacterial infection (SBI) at the time of screening or during the mandatory IVIG treatment period were to be excluded from the study. Other exclusion criteria included lymphoid system malignancy, hyperprolinemia, known allergies or severe reactions to immunoglobulins or other blood products, hypoalbuminemia, protein-losing enteropathies, proteinuria (total urine protein concentration above 0.2 g/L), known hemophilia, or thrombocytopenia (platelet count below or equal to 50×10^9/L). Oral or parenteral steroids were allowed at doses less than 0.15 mg of prednisone equivalent/kg/day. Women who were pregnant, breastfeeding, not using appropriate contraception, or planning a pregnancy during the course of the study were also excluded.

This study was conducted in accordance with the International Conference on Harmonisation Good Clinical Practice guidelines, and the Declaration of Helsinki (2008 version). The study protocol and all other study documents were approved by the relevant independent Ethics Committees. Signed written informed consent (written assent for patients 7 years of age or younger at the time of obtaining the assent) was obtained from the patients or their parents or legally acceptable representatives prior to any study-related activities. This study was registered at ClinicalTrials.gov (study identifier NCT01199705).

Study Design

This prospective, multicenter, open-label, single-arm Phase III study was designed to evaluate the efficacy, tolerability, and safety of IgPro20 in Japanese patients with PID requiring IgG replacement therapy. The primary objective of the study was to assess whether total serum IgG trough levels achieved with preceding IVIG treatment could be sustained upon a dose-equivalent switch to IgPro20.

The study consisted of a screening period, a mandatory IVIG treatment period with three planned infusions at 3- or 4-week intervals at the same dose as the one administered prior to the

study, a 12-week SCIG wash-in/wash-out period, and a 12-week SCIG efficacy period with weekly infusions followed by a completion or discontinuation visit (Fig. 1). For all patients, a viral safety follow-up visit was performed 12–17 weeks after the last SCIG infusion.

Weekly SCIG infusions were allowed to be done at home, except for the first 3 to 8 infusions conducted at the study site. These supervised SCIG infusions were provided to train the patient or their parent or legally acceptable representative who performed the rest of home-based SCIG infusions.

IgPro20 was infused at predefined injection sites on the upper arms, abdomen, thighs, and/or lateral hip recommended by the investigator. The maximum volume per injection site was 25 mL. The actual point(s) of injection could be changed, if needed, with each weekly administration. In case of injection-related local reactions, it was not recommended to use a certain injection site until the local reaction from the previous injection had completely resolved.

To provide a dose-equivalent switch to IgPro20, the initial weekly dose of IgPro20 during the wash-in/wash-out period was calculated as the previous IVIG dose divided by the IVIG dosing interval in weeks. If the IVIG dose had been adjusted during the mandatory IVIG treatment period, the average of the three actual doses was used for calculations. The initial IgPro20 dose could be adjusted, if necessary, based on the IgG trough levels measured during the wash-in/wash-out period, to achieve IgG trough levels of no less than 5 g/L.

The allowed IgPro20 maximal infusion rate for all simultaneously used injection sites during the wash-in/wash-out period (Weeks 1–12) was 25 mL/h or lower. A stepwise increase up to 35 mL/h was allowed for the subsequent infusions at the investigator's discretion, depending on tolerability by the patient.

The main differences between this and the previous two Hizentra® licensing studies [6, 7] were the inclusion of a dedicated IVIG treatment period of 3 months and a pharmacoeconomics analysis.

Efficacy and Safety Assessments

The primary efficacy endpoint was the geometric mean ratio (GMR) of total serum IgG trough levels on IgPro20 therapy versus that achieved during the mandatory IVIG treatment period. Total serum IgG concentration was measured prior to each IVIG infusion and prior to the IgPro20 infusions at Week 1 and every fourth week thereafter. Comparable trough levels were indicated by a GMR close to 1.

Secondary efficacy endpoints included the annualized rates of SBI (according to the pre-specified US Food and Drug Administration [FDA] criteria [16]) and both serious and non-serious infections during the SCIG efficacy period. Patients were required to keep patient diaries, from which the investigators extracted the information necessary to evaluate the number of days missed from work/school or unable to perform normal daily activities due to infections and duration of hospitalization due to infections. Duration of antibiotics use for infection prophylaxis and treatment was assessed from concomitant medications documented in the Case Report Form.

Safety endpoints were the number, rate, severity, and relatedness of any AEs per infusion and patient, local tolerability of subcutaneous infusions, and changes in vital signs (diastolic and systolic blood pressure, heart rate, and body temperature) before and after infusions at the study site. Local tolerability was assessed by patients between 24 and 72 h after the end of infusion and was analyzed descriptively by calculating

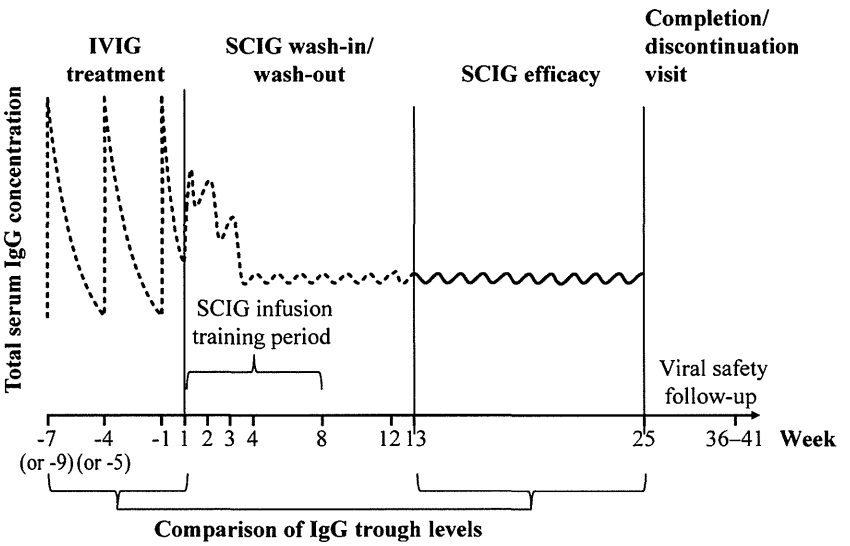

Fig. 1 Study design. Patients received three mandatory IVIG infusions at 3- or 4-week intervals (times for 4-week intervals are shown in brackets), followed by 24 weekly SCIG infusions during the wash-in/wash-out period (Weeks 1–12) and SCIG efficacy period (Weeks 13–25). There were two follow-up visits: completion visit (Week 25) and viral safety follow-up visit 12–17 weeks after completion. The *horizontal curve* schematically represents the expected fluctuations of serum IgG levels before (*dashed curve*) and during the SCIG efficacy period (*solid curve*)

frequency distributions of assessment categories ("very good", "good", "fair", or "poor").

Changes in blood chemistry (albumin, total bilirubin, creatinine, total protein, lactate dehydrogenase, blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase), hematology, and urinalysis and changes in viral safety markers for human immunodeficiency virus (HIV-1 and HIV-2), hepatitis C virus (HCV), and hepatitis B virus (HBV), as compared with baseline assessments, were also recorded.

Statistical Methods

Sample size calculation to assess the GMR of total serum IgG trough levels during IVIG versus SCIG treatment (primary efficacy endpoint) was based upon an assumed residual standard deviation (SD) of 10 %. A 2-sided 90 % confidence interval (CI) was estimated to extend from 0.93 to 1.07 times the GMR if 15 patients were evaluable for the primary analysis. To account for discontinuations, a total of 25 patients were planned for enrollment in the study.

The intention-to-treat (ITT) analysis included data from all patients treated with IgPro20 during the efficacy period. The per-protocol data set (PPS) included all patients who had received at least six doses of IVIG at 3- to 4-week intervals (pre-study and during the IVIG study period) followed by uniform weekly SCIG infusions until at least Week 16 (Fig. 1), with at least one documented total serum IgG trough level in the efficacy period. The safety analysis was based on the "all treated" (AT) data set comprised of all patients who received at least one IgG (IVIG or IgPro20) dose during any study period.

To ensure correct assessment of the GMR, the primary efficacy analysis was based on the PPS, thus excluding patients with dose deviations of >10 %. The GMR (SCIG efficacy period versus mandatory IVIG treatment) with a 2-sided 90 % CI was calculated using mean log-transformed IgG trough levels averaged by patient and treatment period. Additionally, descriptive statistics were calculated for all IgG trough levels by visit and by treatment period. Analyses of secondary efficacy variables were based on the data set used for ITT analysis and the PPS.

Analyses of safety endpoints were based on the AT set. Baseline measurements refer to the sample taken prior to first mandatory IVIG infusion.

Results

Patients

A total of 25 patients were screened and enrolled into the study at nine sites throughout Japan. One patient discontinued

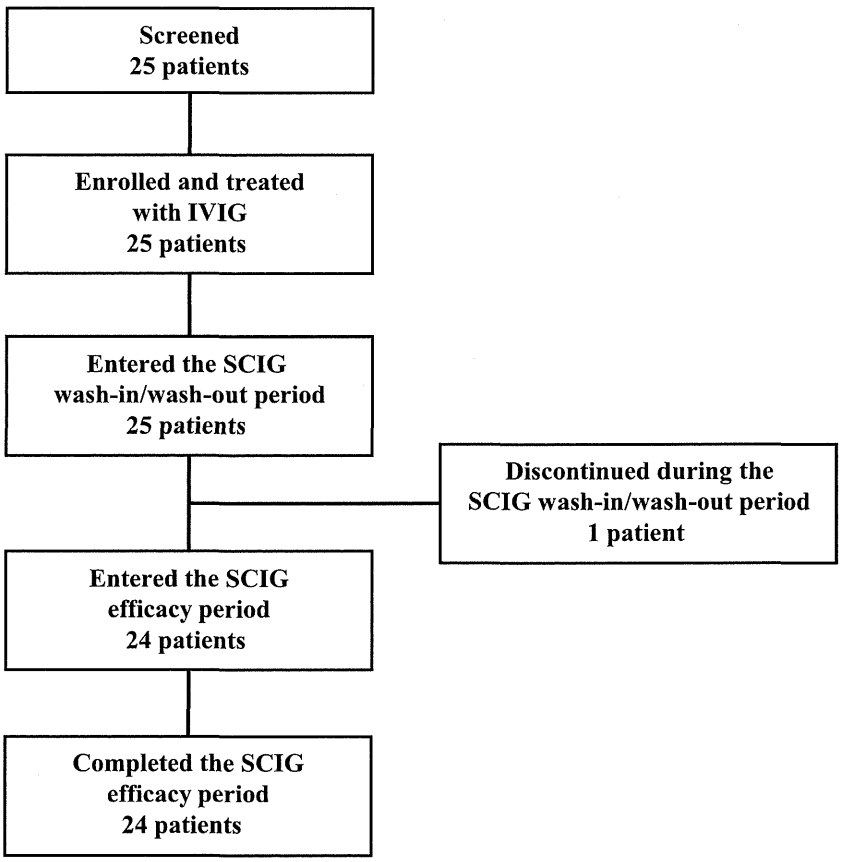

Fig. 2 Patient disposition. All 25 screened patients were enrolled into the study and received mandatory IVIG treatment. One patient discontinued from the study during the SCIG wash-in/wash-out period, leaving 24 patients who completed the study

from the study during the wash-in/wash-out period due to relocation, leaving 24 patients who completed the study (Fig. 2).

The mean (SD) age of the patients was 20.5 (13.5) years; 11 patients were ≤16 years of age. Fifteen patients (62.5 %) were male (Table I).

The most common PID conditions were X-linked agammaglobulinemia (XLA; 12 patients [50 %], including one well-documented rare female case [17]) and common variable immunodeficiency (CVID; 10 patients [41.7 %]).

Study Drug Administration

All 25 patients received the intended three IVIG infusions and at least one dose of IgPro20, of which 24 patients received the intended 12 infusions during the SCIG wash-in/wash-out period and another 12 infusions during the efficacy period.

The mean (SD) of individual median weekly IgPro20 doses during the SCIG efficacy period was 87.8 (35.2) mg/kg body weight (bw; Table II). The mean (SD) of the weekly equivalents of individual IVIG doses was 77.3 (30.5) mg/kg bw. An increase from the planned dose by >10 % at any time during the study was documented in 12 patients (48 %).

Table I Demographic characteristics of patients

Patients	ITT	PPS
Total number of patients	24	21
Gender, n (%)		
Male	15 (62.5)	14 (66.7)
Female	9 (37.5)	7 (33.3)
Age [years], median (range)	17.5 (3–58)	19.0 (3–58)
Age group, n (%)		
<2 years	0	0
≥2 to <12 years	7 (29.2)	4 (19.0)
≥12 to <16 years	4 (16.7)	4 (19.0)
≥16 to <65 years	13 (54.2)	13 (61.9)
≥65 years	0	0
Asian, n (%)	24 (100)	21 (100)
Body weight [kg], median (range)	44.8 (13–105)	48.9 (13–105)
BMI [kg/m^2], median (range)	18.2 (15–33)	18.2 (15–33)
Type of PID, n (%)		
CVID	10 (41.7)	9 (42.9)
XLA	12 (50.0)	11 (52.4)
ARAG	1 (4.2)	0
VHyper IgM syndrome	1 (4.2)	1 (4.8)

ARAG autosomal recessive agammaglobulinemia, *BMI* body mass index, *CVID* common variable immunodeficiency, *ITT* intention-to-treat, *n* number of patients, *PID* primary immunodeficiency, *PPS* per-protocol data set, *XLA* X-linked agammaglobulinemia

Table II Weekly IgG doses by treatment period (ITT)

Treatment period	IVIG[a]	SCIG wash-in/wash-out	SCIG efficacy
Total number of infusions	72	288	288
IgG dose, mg/kg bw			
Mean (SD)	77.3 (30.5)	82.2 (33.4)	87.8 (35.2)
Median	73.00	72.98	77.82
Range, min–max	21.5–144.3	26.4–177.8	26.7–172.7

[a] Weekly equivalent dose across both application schedules (every 3 weeks and every 4 weeks) was calculated based on individual infusions

bw body weight, *ITT* intention-to-treat, *IVIG* intravenous immunoglobulin, *SCIG* subcutaneous immunoglobulin, *SD* standard deviation

The mean (range) of the individual infusion rates was 22.90 mL/h (13–25 mL/h) during the SCIG wash-in/wash-out period and 25.35 mL/h (12–35 mL/h) during the SCIG efficacy period. The majority of infusions (74.0 %) during the SCIG efficacy period were home-based.

Efficacy

Primary Efficacy Endpoint

The primary objective of the study was met, as the GMR of serum IgG trough levels after dose-equivalent switch from IVIG to SCIG was close to 1, with the CI within the accepted equivalence range of 0.80 to 1.25 (1.09 in the PPS [90 % CI: 1.06–1.13]). The mean (SD) IgG trough levels slightly increased from 6.53 (1.40) g/L in the IVIG period to 7.15 (1.51) g/L in the SCIG efficacy period (Fig. 3).

The ITT analysis confirmed these results.

Secondary Efficacy Endpoints

No SBIs were reported at any time during the study.

Rates of infection were maintained at a low level during the SCIG efficacy period, with 11 patients (52.4 %) experiencing an infection (annualized rate of 2.98 infections per patient/year; Table III). Seven patients (33.3 %) missed a total of 19 days from school/work/kindergarten due to infection (3.48 days per patient/year). One patient (4.8 %) was hospitalized during the SCIG efficacy period due to bacterial infection of moderate severity, for a total of 3 days (0.55 days of hospitalization per patient/year); for details see the Safety section.

A total of 16 patients (76.2 %) were treated with antibiotics on 844 days during the SCIG efficacy period, resulting in an annualized rate of 167.42 days of antibiotic use per patient/year. Antibiotics were used for both treatment of an AE (10 patients [47.6 %]) and for prophylaxis (5 patients [23.8 %]).

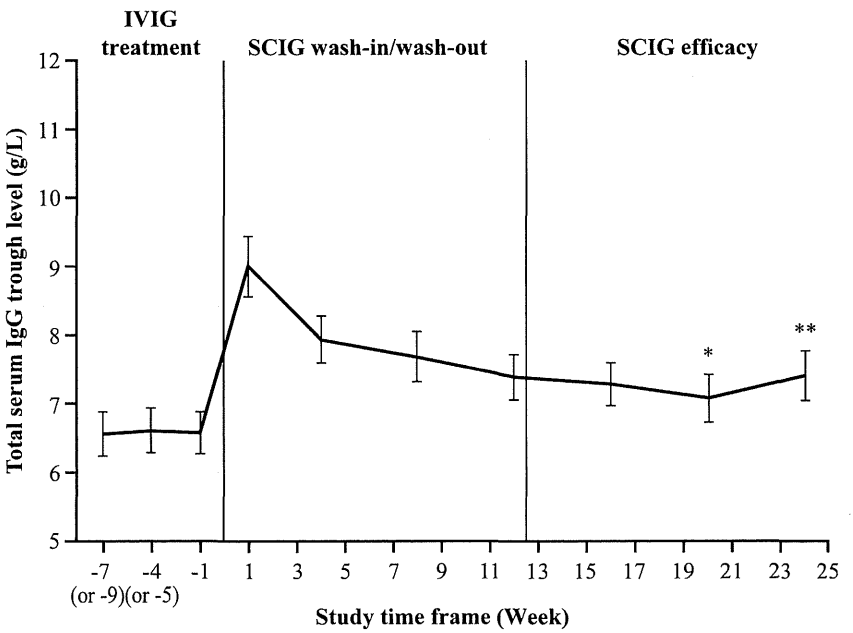

Fig. 3 Mean total serum IgG trough levels (PPS). Mean total serum IgG trough levels are shown for the PPS (N=21). The number of patients with available IgG levels differed from the original patient number in the PPS at Week 20 (N=20; *) and Week 24 (N=18; **). *Error bars* represent the standard error of the mean

Safety

Overall Adverse Events

In the SCIG wash-in/wash-out and SCIG efficacy periods, 24 of the 25 patients (96.0 %) had a total of 269 AEs, with an overall AE rate of 0.461 AEs per infusion. All AEs were of mild or moderate severity, with only 5 AEs (0.009 AEs per infusion) in 3 patients (12.0 %) being of moderate severity. The most common AEs were local reactions (80.0 %), followed by infections, the most common being nasopharyngitis (44.0 %), upper respiratory tract infection (20.0 %), and gastroenteritis (16 %). All other AEs each occurred in less than 4 patients (≤12 %).

During the mandatory IVIG treatment period, 22 patients (88.0 %) experienced 49 AEs (0.653 AEs per IVIG infusion).

Temporally Associated and Related Adverse Events

During SCIG treatment, 21 patients (84.0 %) had a total of 175 AEs that were considered by the investigator at least possibly related to the study drug (0.300 AEs per infusion) and 23 patients (92.0 %) experienced 203 AEs that were temporally associated, i.e., occurred within 72 h after an SCIG infusion (0.348 AEs per infusion; Table IV). The majority of the causally-related AEs (91.3 %) and both causally- and temporally-related AEs (91.7 %) were mild local reactions.

In the IVIG treatment period, 1 patient (4.0 %) had at least 2 possibly related AEs (malaise and pyrexia; 0.027 AEs per infusion) and 10 patients (40.0 %) had 12 temporally associated AEs (0.160 AEs per infusion).

None of the infusions was stopped due to an AE. The IgPro20 infusion rate was increased at the investigator's discretion in 8 patients. None of the AEs reported in these patients could be associated with the increased infusion rate.

Serious Adverse Events

During the SCIG treatment, one serious AE (SAE) of moderate severity was reported (0.002 SAEs per SCIG infusion) that

Table III Summary of secondary efficacy endpoints (PPS)

Secondary efficacy endpoint	Number of patients (%)	Number of events/days (annualized rate)
Total number of patients or exposure days (diary days[a])	21	1,840 (1,990)
SBIs	0	0
Infection episodes	11 (52.4)	15 (2.98)
Days with antibiotics for infection prophylaxis	5 (23.8)	422 (83.71)
Days with antibiotics for infection treatment	13 (61.9)	458 (90.85)
Days hospitalized due to infections[a]	1 (4.8)	3 (0.55)
Days missed from work/school or unable to perform normal activities due to infections[a]	7 (33.3)	19 (3.48)

[a] As per "diary days" based on analysis of patients' diaries. *SBI* serious bacterial infection; *PPS* per-protocol data set

Table IV Most common related AEs during the entire SCIG treatment period (SCIG wash-in/wash-out and SCIG efficacy periods) by preferred term (AT)

Preferred term	At least possibly related		At least possibly related and temporally associated events (72 h)	
	Number of patients (%)	Number of events (rate per infusion)	Number of patients (%)	Number of events (rate per infusion)
Total number of patients or infusions	25	584	25	584
Any preferred term	21 (84.0)	175 (0.300)	21 (84.0)	170 (0.291)
Local reactions[a]	20 (80.0)	160 (0.274)	20 (80.0)	156 (0.267)
Skin and subcutaneous tissue disorders	2 (8.0)	9 (0.015)	2 (8.0)	9 (0.015)
Gastrointestinal disorders	1 (4.0)	1 (0.002)	1 (4.0)	1 (0.002)
Investigations	1 (4.0)	1 (0.002)	0 (0.0)	0 (0.000)
Vascular disorders	1 (4.0)	1 (0.002)	1 (4.0)	1 (0.002)

[a] Based on 16 MedDRA preferred terms

AE adverse event, *AT* all treated data set, *SCIG* subcutaneous immunoglobulin

was considered by the investigator unrelated to study drug. A 22-year-old male with XLA experienced bacterial infection that was reported as an SAE because of the necessity of hospitalization. The patient was treated with antibacterials and the infection resolved after 15 days. This infection was not considered an SBI, as it did not meet the pre-specified US FDA criteria. No SAEs were reported during the IVIG treatment period.

There were no deaths or AEs resulting in discontinuation of treatment in this study.

Local Reactions

Local tolerability of 85.4 % of SCIG infusions was assessed by the patients as "very good" or "good". In no case was the local tolerability assessed as "poor".

AEs of local reactions occurred in 20 patients (80.0 %), with an overall rate of 0.277 events per infusion. Almost all local reactions (97.5 %) were temporally associated with SCIG infusion. The rate of local reactions decreased over time from 0.389 events per infusion during the SCIG wash-in/wash-out period to 0.163 events per infusion during the SCIG efficacy period. The overall rate of local reactions related to home-based infusions during the efficacy period was comparable with that related to infusions at investigational site (0.178 events per infusion versus 0.120 events per infusion, respectively).

Vital Signs, Laboratory Parameters, and Viral Safety

No consistent or clinically relevant changes in vital signs were reported in this study.

Median values and ranges of hematology, blood chemistry, and urinalysis did not show any relevant changes over time.

Viral safety screening for HIV-1, HIV-2, HCV, and HBV found no positive viral markers at either baseline or 12–17 weeks following the final SCIG infusion.

Discussion

Primary efficacy analysis of this study demonstrated that IgPro20 administered by the subcutaneous route in uniform weekly doses was an effective treatment in both adult and pediatric Japanese patients with PID. A dose-equivalent switch to SCIG 20 % was effective in maintaining total serum IgG trough levels equal to or above those achieved on the previous IVIG therapy (Fig. 3). The annualized rate of infection during the SCIG efficacy period (2.98 infections/patient/year) was in line with that observed in previous studies in Europe and in the US (2.76–5.18 infections/patient/year), as were the number of days missed from school/work/daycare and days spent in hospital [6, 7, 12]. The mean weekly dose of IgPro20 during the SCIG efficacy period was lower in the Japanese population than that seen in Europe and, in particular, the US [6, 7], probably due to the specifics of local treatment practices including limits on maximum dose allowed per patient.

This is the first prospective study of SCIG to incorporate a mandatory IVIG treatment period into the trial design, allowing for a more stringent comparison of SCIG and IVIG treatments than previous IgPro20 studies. As all patients had received at least 3 IVIG infusions at a stable dose before enrolling in the study, their IgG levels were expected to be at steady state by the end of the mandatory IVIG period. A 12-week SCIG wash-in/wash-out period ensured that the IVIG treatment did not affect serum IgG trough levels during the steady state SCIG efficacy period. SCIG dose adjustments during the wash-in/wash-out period were allowed, but were unlikely to affect the outcome of the study, as the minimal target serum IgG trough level (5.0 g/L) was lower than that achieved during the IVIG treatment period (6.53 g/L).

The male/female imbalance in the patient demographics can be explained by a large share of XLA patients in the study population. Of interest, this subgroup included one well-documented case of XLA in a female patient [17].

Higher rates of antibiotic use in this study compared with the same outcome in SCIG studies conducted outside Japan are likely associated with generally higher administration of antibiotics in Japan [18–20].

IgPro20 was well tolerated. No SAEs related to its administration were reported during the study. One SAE (bacterial infection; etiologic agent not identified) of moderate severity reported during the SCIG efficacy period occurred due to accidental infection and was considered unrelated to the study drug.

The overall incidence of AEs including local reactions during the SCIG wash-in/wash-out and SCIG efficacy periods (0.461 AEs per patient/year affecting 96 % of patients) was comparable with the overall AE rates in other SCIG studies in Europe and in the US (0.288–0.773 AEs per patient/year affecting 98–100 % of patients) [6, 7]. Mild or moderate local reactions (swelling, soreness, redness, and induration) are generally expected when relatively large volumes of IgG are infused by the subcutaneous route. The overall rates of local reactions reported in this study were in line with previous SCIG studies [6, 7, 11, 12].

Conclusions

Weekly SCIG treatment with Hizentra® was effective in pediatric and adult Japanese patients with PID requiring IgG replacement therapy. The treatment was well tolerated and demonstrated a highly favorable risk-benefit profile.

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