CREATING AWARENESS FOR PRIMARY IMMUNODEFICIENCIES IN THE SOUTHEAST AND EAST ASIA REGIONS

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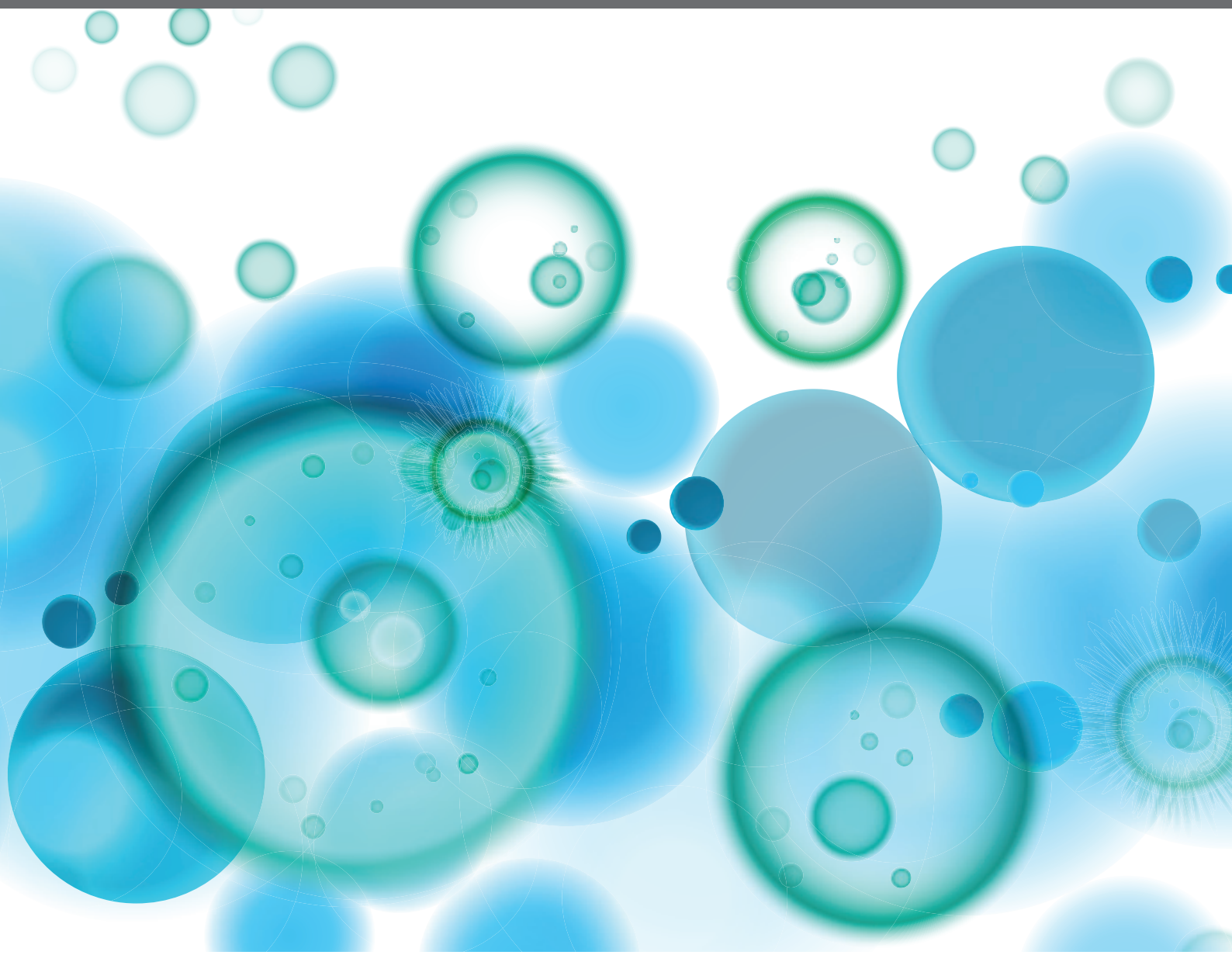

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CREATING AWARENESS FOR PRIMARY IMMUNODEFICIENCIES IN THE SOUTHEAST AND EAST ASIA REGIONS

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EDITORIAL
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Editorial: Creating Awareness for Primary Immunodeficiencies in the Southeast and East Asia Regions

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Editorial on the Research Topic

Creating Awareness for Primary Immunodeficiencies in the Southeast and East Asia Regions

Primary immunodeficiencies (PIDs) are genetic disorders characterized by inborn errors of immunity with various clinical manifestations, including increased susceptibility to infections, autoinflammation, autoimmunity, lymphoproliferation, allergy, and predisposition to malignancy. PID is an emerging disease in several parts of Asia, including East Asia (EA) and Southeast Asia (SEA). Reports from SEA and EA regions demonstrates that PID is more common than usually thought and constitutes an evolving disease that needs to be addressed. However, these disorders receive less attention compared to the other established diseases and lack awareness; therefore, PIDs are often under-reported. The published reports of PIDs in SEA seem to be extremely less than actual case prevalence. This Research Topic is intended to summarize the recent progress in understanding PIDs in SEA and EA regions.

Basic immunology is essential for the understanding of PIDs. DOCK8 deficiency is a combined immunodeficiency characterized by allergic diseases. Jiang et al. revealed that patients with DOCK8 deficiency and the *Dock8* knock-out mouse model had defects in IL-10-producing regulatory B (Breg) cells. In addition, IL-21 restored the function of Bregs in the *Dock8*-deficient mouse.

In patients with Wiskott-Aldrich syndrome (WAS), T-cell receptor (TCR) diversity was severely impaired. Li et al. demonstrated that WASp deficiency selectively affected the TCR diversity of different memory T-cell subsets using *Was* knock-out mouse model.

Infections are the primary cause of morbidity and mortality in patients with PID. Tang et al. demonstrated that metagenomic next-generation sequencing successfully identified microorganism compared with the conventional microbiological test.

A nationwide survey of each country was published on this Research Topic. DNA ligase IV (LIG4) deficiency is an extremely rare PID. Luo et al. described that in patients with LIG4 deficiency, p.R278L variant is observed in most patients and this variant has a founder effect in the Chinese population.

Gastrointestinal (GI) symptoms are often observed in patients with PID. Therefore, Kim et al. performed a nationwide survey of GI symptoms in Korean patients with PIDs. Of 165 patients, 55 (33.1%) showed GI manifestations.

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A nationwide survey of PIDs was performed thrice in Japan. Takada summarizes these survey results and focuses on IRAK4 deficiency as an example of creating awareness for its appropriate management.

The Primary Immunodeficiency Database in Japan (PIDJ) is a registry of Japanese patients with PIDs, which was established in 2007. Mitsui-Sekinaka et al. reported that 4,481 patients had been enrolled in PIDJ. In 2017, the Japanese Society for Immunodeficiency and Autoinflammatory Diseases (JSIAD) was launched. Furthermore, the PIDJ was upgraded to "PIDJ ver.2" in 2019, supported by JSIAD to promote epidemiological studies, genetic analysis, and pathogenetic evaluation for PIDs and autoinflammatory diseases.

Serum immunoglobulin (Ig) measurements are not widely accessible in developing countries. Suratannon et al. reported a simple prediction model using serum globulin to predict the likelihood of low IgG levels in children.

PIDs may have phenotypic differences in the SEA, EA, and other regions. For example, Kadowaki et al. described a characteristic of A20 haploinsufficiency (HA) in Asia. HA20 is an early-onset autoinflammatory disease resembling Behçet's disease (BD). Patients with HA20 in the EA developed recurrent fever more frequently than those in other regions but were less likely to develop BD symptoms.

Some patients with early-onset inflammatory bowel disease (IBD) are associated with PIDs. Sasahara et al. review PID-associated early-onset IBD in SEA and EA. The prevalence of PIDs associated with IBD was higher than in western countries. A Japanese cohort revealed that patients with XIAP and IL10RA deficiencies were recurrently observed; however, patients with IL10R deficiency were preferentially reported in China. Therefore, a comprehensive molecular diagnosis should be applied to screen for PID-associated IBD in SEA and EA to improve the prognosis.

Chronic granulomatous disease (CGD) is a PID characterized by recurrent bacterial and fungal infections and it is inherited as an X-liked (XL) or autosomal recessive (AR) mode. Chiu et al. revealed phenotypic differences between XL- and AR-CGD in Asia and Africa. XL-CGD patients had a younger age of onset, referral, and diagnosis than AR-CGD patients. In addition, patients with XL-CGD had more recurrent and severe infections than patients with AR-CGD.

A couple of comprehensive reviews of PIDs are included in this Research Topic. Miyazawa and Wada discussed the most recent research on reversion mosaicism in PIDs. Several mechanisms can mediate the somatic reversion of inherited variants. Furthermore, revertant cells with wild-type function may be associated with milder phenotypes in PID patients with reversion mosaicism.

IKAROS and CTLA4 deficiencies are PIDs showing similar clinical phenotypes, including hypogammaglobulinemia and autoimmune disease (AD). Hoshino et al. performed a systematic literature review of these diseases. IKAROS deficiency revealed that AD and hypogammaglobulinemia develop in that order, and AD resolves before the onset of hypogammaglobulinemia. Conversly, these observations were not found in CTLA4 deficiency.

PIDs frequently affect the endocrine system. Takasawa et al. comprehensively reviewed the understanding of endocrine disorders clinical features and pathophysiology in PIDs.

This Research Topics included interesting case reports. For example, Ripen et al. reported on a Malaysian girl affected with both Williams-Beuren syndrome and CGD (*NCF1*). Whole-exome sequencing revealed a hemizygous deletion of 1.53Mb on chromosome 7q11.23.

Inaba et al. presented a report of a Japanese boy with NEMO deficiency caused by a novel hypomorphic variant of *IKBKG*. He also stated that his deceased maternal uncles had the same *IKBKG* variants using preserved umbilical cord blood cells.

Severe combined immunodeficiency (SCID) is a PID primarily due to impaired lymphocyte differentiation. Hematopoietic cell transplantation (HCT) is a curable treatment, but the outcomes of HST for SCID are poor when patients have active infections. Tanita et al. reported that five Japanese patients with SCID were infected with rotavirus infection derived from the oral rotavirus vaccine.

Li et al. described six Chinese patients with type I interferonopathy who were treated with Janus kinase (JAK) inhibitors. The JAK inhibitors (baricitinib and tofacitinib) are promising drugs for patients with type I interferonopathy including STING-associated vasculopathy with onset in infancy, Aicardi-Goutières syndrome, and spondyloenchondrodysplasia with immune dysregulation.

AUTHOR CONTRIBUTIONS

HK wrote the manuscript. II and XZ provided critical discussion. IHI and XZ edited the manuscript. All authors contributed to the article and approved the submitted version.

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Needs for Increased Awareness of Gastrointestinal Manifestations in Patients With Human Inborn Errors of Immunity

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The gastrointestinal (GI) tract is frequently affected by inborn errors of immunity (IEI), and GI manifestations can be present in IEI patients before a diagnosis is confirmed. We aimed to investigate clinical features, endoscopic and histopathologic findings in IEI patients. This was a retrospective cohort study conducted from 1995 to 2020. Eligible patients were diagnosed with IEI and had GI manifestations that were enough to require endoscopies. IEI was classified according to the International Union of Immunological Societies classification. Of 165 patients with IEI, 55 (33.3%) had GI manifestations, and 19 (11.5%) underwent endoscopy. Among those 19 patients, nine (47.4%) initially presented with GI manifestations. Thirteen patients (68.4%) were male, and the mean age of patients 11.5 ± 7.9 years (range, 0.6 – 26.6) when they were consulted and evaluated with endoscopy. The most common type of IEI with severe GI symptoms was "Disease of immune dysregulation" (31.6%) followed by "Phagocyte defects" (26.3%), according to the International Union of Immunological Societies classification criteria. Patients had variable GI symptoms such as chronic diarrhea (68.4%), hematochezia (36.8%), abdominal pain (31.6%), perianal disease (10.5%), and recurrent oral ulcers (10.5%). During the follow-up period, three patients developed GI tract neoplasms (early gastric carcinoma, mucosa associated lymphoid tissue lymphoma of colon, and colonic tubular adenoma, 15.8%), and 12 patients (63.2%) were diagnosed with inflammatory bowel disease (IBD)-like colitis. Investigating immunodeficiency in patients with atypical GI symptoms can provide an opportunity for correct diagnosis and appropriate disease-specific therapy. Gastroenterologists and immunologists should consider endoscopy when atypical GI manifestations appear in IEI patients to determine if IBD-like colitis or neoplasms including premalignant and malignant lesions have developed. Also, if physicians in various fields are better educated about IEI-specific complications, early diagnosis and disease-specific treatment for IEI will be made possible.

Keywords: inborn errors of immunity, primary immunodeficiencies, gastrointestinal, endoscopy, malignancy, inflammatory bowel disease

INTRODUCTION

Human inborn errors of immunity (IEI) are a heterogeneous and increasing set of more than 350 genetic disorders affecting the development and/or the function of various components of the innate and adaptive immune system (1). Although respiratory symptoms seem to be the most common manifestation of IEI, the gastrointestinal (GI) system is the second most common site of complications.

Recent studies revealed that the prevalence of GI manifestations in patients with IEI varies from 5% to 50% and depends on the specific immunodeficiencies in each patient (2). This is partially because the gut-associated lymphoid tissue is the largest lymphoid organ in the body and is thus frequently affected by states of immune deficiency (2, 3). In addition, the mucosal immune system of the GI tract is carefully regulated to maintain homeostasis in the face of exposure to bacterial antigens, viruses, fungi, and dietary antigens, all of which exist in close proximity to a large reservoir of immune cells, such as lymphocytes, macrophages, and dendritic cells. Impairment of the regulatory mechanisms that maintain homeostasis between active immunity and tolerance in the GI tract may cause mucosal inflammation and damage (4). Therefore, the GI tract, which contains an abundance of lymphoid tissue, is frequently affected in IEI, and GI manifestations can be present in patients with IEI before diagnosis.

The GI manifestations of IEI are divided into 4 groups: infection, malignancy, inflammation, and autoimmunity (5–7). The GI symptoms in patients with IEI may mimic those of other GI diseases, such as inflammatory bowel disease (IBD), although the pathophysiology, treatment strategy and response to conventional treatment are distinct. Thus, patients presenting with atypical GI disease and/or failure to respond to conventional therapy should be evaluated for the possibility of underlying IEI and appropriate treatment should be initiated.

The GI manifestations of IEI have not received much attention from physicians in various fields. We aimed to investigate the characteristics of GI symptoms and the endoscopic and histopathologic findings in IEI patients who had been evaluated with endoscopies.

MATERIALS AND METHODS

This was a retrospective cohort study conducted at the Department of Pediatrics of Samsung Medical Center from December 1995 to July 2020. Patients were eligible if they had IEI and developed GI manifestations that were severe enough to require endoscopic evaluation during the study period (**Figure 1**). Severe GI manifestations are GI bleeding, abdominal pain with signs or symptoms suggesting serious organic disease (e.g., weight loss, anorexia, anemia) associated with significant morbidity (e.g., prolonged hospitalization, limitation of usual activities), and chronic diarrhea of unexplained origin that lasts for more than

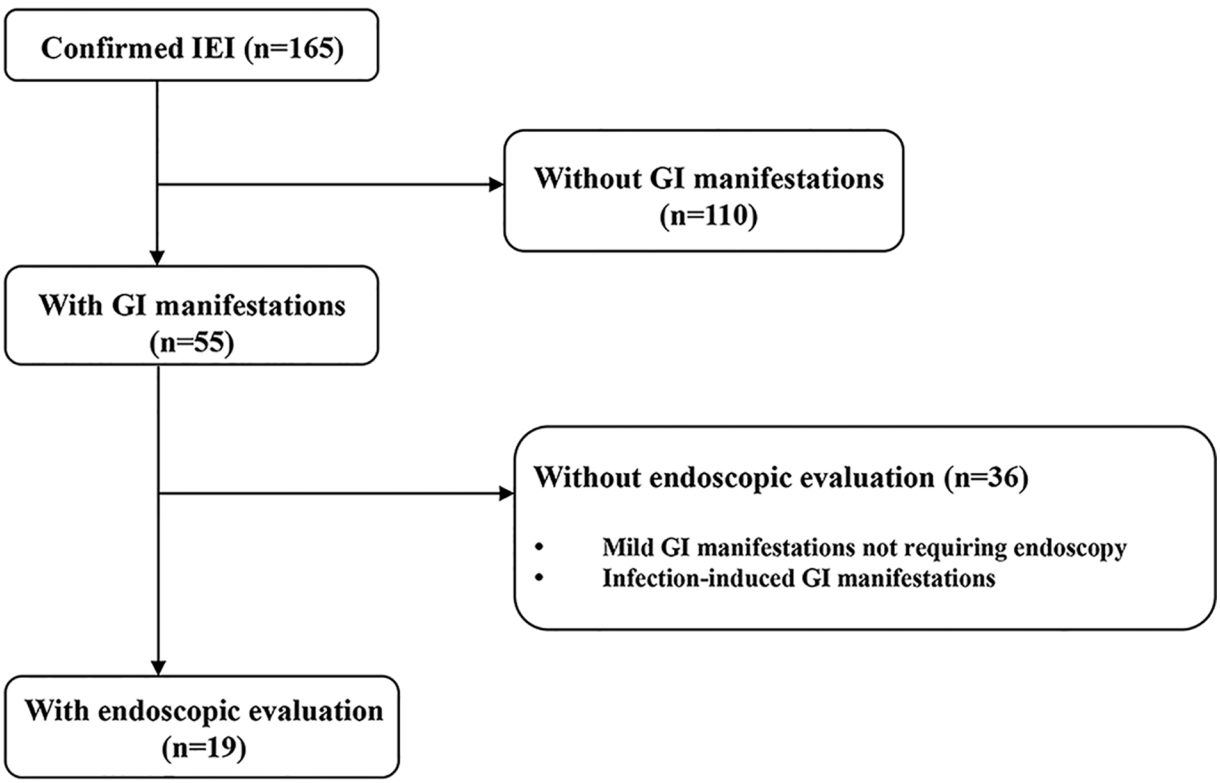

FIGURE 1 | Study flow diagram. IEI, inborn errors of immunity; GI, gastrointestinal.

2–4 weeks. Cases with worsening GI symptoms that were caused by infection were excluded. The patients were divided according to the updated phenotypical classification for IEI (1), established by the International Union of Immunological Societies Expert Committee and were analyzed retrospectively. Baseline demographic and clinical data at initial endoscopy, including sex, age, growth indicators, GI manifestations, and other organ involvement in IEI were collected from electronic medical records. During the endoscopic evaluation, biopsies were taken from abnormal lesions to evaluate histopathologic alterations from the esophagus to the duodenum in esophagogastroduodenoscopy (EGD) and from the terminal ileum to rectum in ileocolonoscopy. For infants with severe GI manifestations, sigmoidoscopy was performed from the descending colon to rectum. Macroscopic and microscopic findings of the GI tract were analyzed. Continuous variables are presented as medians and interquartile ranges (IQRs) for non-normally distributed data or as means and standard deviations for normally distributed data. Categorical variables are presented as number and proportion. All statistical analyses were carried out using Rex (Version 3.0.3, RexSoft Inc., Seoul, Korea).

RESULTS

Patient Characteristics

From December 1995 to July 2020, a total of 165 patients were diagnosed with IEI after immunological and/or genetic investigations. Amongst these 165 patients, the most common type of IEI is "Combined immunodeficiency with associated or syndromic features" (50/165, 30.3%), followed by "Predominantly antibody deficiencies" (34/165, 20.6%), "Congenital defects of phagocyte number or function" (30/165, 18.2%), "Diseases of immune dysregulation" (27/165, 16.4), "Defects in intrinsic and innate immunity" (7/165, 4.2%), "Complement deficiencies" (2/165, 1.2%), "Auto-inflammatory disorders" (1/165, 0.6%) and "Phenocopies of IEI" (1/165, 0.6%). Four patients were unidentified IEI (4/165, 2.4%).

Fifty-five patients (55/165, 33.3%) had GI manifestations, and 19 patients (19/165, 11.5%) received endoscopy. The most common type of IEI that involved severe GI symptoms was "Disease of immune dysregulation" (6/19, 31.6%) followed by "Phagocyte defects" (5/19, 26.3%) (**Table 1**). In 47.4% (9/19) of patients who were evaluated with endoscopy, severe and chronic GI symptoms preceded the diagnosis of IEI. Among the patients who developed GI manifestations before the diagnosis of IEI, the groups that accounted for the largest proportion was "Disease of immune dysregulation" (3/9, 33.3%) and "Phagocyte defects" (3/9, 33.3%). In particular, in patients who developed GI manifestations before IEI diagnosis, the average time from GI symptoms onset to IEI diagnosis was 2.6 years (range 0.2–14.4). Except for one patient, the diagnosis of IEI was not delayed as eight patients initially presented clinical symptoms similar to those of IBD.

Among patients who received endoscopy, thirteen patients (13/19, 68.4%) were male, and the mean age of all patients was 11.5 ± 7.9 years (range, 0.6–26.6) at the time of consultation and endoscopic evaluation. Patients presented with varied GI symptoms at the time of the initial endoscopic evaluation, including diarrhea (13/19, 68.4%), hematochezia (7/19, 36.8%), abdominal pain (6/19, 31.6%), perianal fistula and/or abscess (2/19, 10.5%) and recurrent oral ulcers (2/19, 10.5%). Furthermore, growth impairment was observed in six patients (6/19, 31.6%).

TABLE 1 | Distribution of IEI patients with severe GI symptoms enough to require endoscopy.

Group of Immunodeficiencies	Total, *n* (%)	Patients with GI symptoms before IEI diagnosis, *n* (%)	Patients with GI symptoms after IEI diagnosis, *n* (%)
Total	**19 (100.0)**	**9 (47.4)**	**10 (52.6)**
Predominantly antibody deficiencies	**4 (21.1)**	**1 (5.3)**	**3 (15.8)**
Common variable immunodeficiency	2	0	2
Activated PI3k delta syndrome	2	1	1
Congenital defects of phagocyte number or function	**5 (26.3)**	**3 (15.8)**	**2 (10.5)**
Chronic granulomatous disease	4	2	2
Glycogen storage disease type 1b	1	1	0
Disease of immune dysregulation	**6 (31.6)**	**3 (15.8)**	**3 (15.8)**
CTLA4 deficiency	2	1	1
IPEX syndrome	1	1	0
XIAP	1	1	0
X-linked lymphoproliferative syndrome	2	0	2
Defects in intrinsic and innate immunity	**2 (10.5)**	**1 (5.2)**	**1 (5.3)**
Osteopetrosis	1	1	0
STAT1 gain of function	1	0	1
Autoinflammatory disorders	**2 (10.5)**	**1 (5.3)**	**1 (5.2)**
Blau syndrome	1	1	0
Chronic recurrent multifocal osteomyelitis	1	0	1

IEI, inborn errors of immunity; GI, gastrointestinal; PI3k, phosphoinositide 3 kinase; CTLA4, cytotoxic T lymphocyte-associated antigen-4; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked; XIAP, X-linked inhibitor of apoptosis; STAT1, signal transducer and activator of transcription 1.

In 10 patients who developed GI manifestations after the diagnosis of IEI, the elapsed time between the diagnosis of IEI and GI symptoms onset was 4.7 years (range 0.1–8.7 years). During the follow-up period, three patients (3/19, 15.8%) developed GI tract neoplasms (early gastric carcinoma, MALT (mucosa associated lymphoid tissue) lymphoma of colon and colonic tubular adenoma), and 12 patients (12/19, 63.2%) were diagnosed with IBD-like colitis.

Regarding therapies, patients diagnosed with activated PI3K δ syndrome (APDS), cytotoxic T lymphocyte-associated antigen 4 (CTLA 4) deficiency received therapies specifically oriented in the mechanism of the underlying immunological disease; sirolimus for APDS and CTLA4-Ig (abatacept) for CTLA 4 deficiency. Of two patients diagnosed with CTLA 4 deficiency, one received subtotal gastrectomy for early gastric carcinoma, and one received abatacept. In addition, six patients diagnosed with chronic granulomatous disease (CGD), glycogen storage disease (GSD) type 1b, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX syndrome), X-linked inhibitor of apoptosis protein deficiency (XIAP) underwent hematopoietic cell transplantation (HCT).

The patients with IBD-like colitis were treated with mesalazine (n = 8), azathioprine (n = 2), methotrexate (n = 2), steroids (n = 2), or anti-TNFα (n = 4) according to the IBD treatment guidelines (8). Three patients (CRMO, XIAP, and Osteopetrosis) responded to anti-TNFα treatment with IBD symptom improvement. Three patients (two patients with CGD, one patient with IPEX) underwent HCT to treat both IEI and IBD-like colitis. One patient with CTLA4 deficiency received abatacept and showed a dramatic response. Other baseline characteristics of the study subjects are summarized in **Table 2** and detailed clinical characteristics of 19 patients are presented in **Table 3**.

Endoscopic Findings

Nineteen patients with IEI underwent a total of 111 endoscopies during the follow-up period. These included 57 EGDs, 53 ileocolonoscopies and one sigmoidoscopy. **Table 4** gives the details of the endoscopic findings of all 19 patients. Endoscopic examination of the esophagus was normal in 14 patients (14/19, 73.7%), and five patients exhibited abnormality in the esophagus such as esophagitis, esophageal ulcer, and esophageal varix. Chronic gastritis (8/19, 42.1%) was the most common macroscopic finding in the stomach, and early gastric carcinoma was diagnosed in one patient with CTLA 4 deficiency (**Figure 2A**). Duodenal ulcer and lymphoid hyperplasia of the duodenum were each identified in four patients (4/19, 21.1%).

On ileocolonoscopy, premalignant and malignant lesions were found in the lower GI tracts of two patients: colonic tubular adenoma in a common variable immunodeficiency (CVID) patient and MALT lymphoma in an APDS patient proved by immunoglobulin heavy chain gene rearrangement (**Figures 2B, C**).

Features of IBD-like colitis were found in 12 patients (12/19, 63.2%): five patients were diagnosed with congenital defects of phagocyte number or function (CGD, GSD type 1b); three patients with diseases of immune dysregulation (IPEX syndrome, XIAP deficiency (**Figure 3A**), CTLA4 deficiency); two patients with auto-inflammatory disorders [Blau syndrome;

TABLE 2 | Baseline characteristics of IEI patients with GI manifestations required endoscopic evaluation.

	Total (n = 19)
Males, *n* (%)	13 (68.4)
Age at diagnosis *of* IEI, *years* (IQR)	5.1 (1.4, 10.8) (range, 0.4–24.5)
Age at initial GI symptoms, *years*	9.3 ± 7.0 (range, 0–24.9)
Age at initial endoscopy, *years*	11.5 ± 7.9 (range, 0.6–26.6)
GI manifestations before IEI diagnosis, *n* (%)	9 (47.4)
Observational duration, years (IQR)	5.1 (1.5–11.4)
Body weight z-score	-1.3 ± 1.5
Height z-score	-1.1 ± 1.3
BMI z-score	-0.6 ± 1.8
Growth impairment, *n* (%)	6 (31.6)
GI manifestations, n (%)	
Abdominal pain	6 (31.6)
Diarrhea	13 (68.4)
Hematochezia	7 (36.8)
Recurrent oral ulcer	2 (10.5)
Perianal fistula/abscess	2 (10.5)
Premalignant and malignant lesions during follow-up	3 (15.8)
IBD-like colitis, *n* (%)	12 (63.2)
Other organ involvement*, *n* (%)	
Lung	6 (31.6)
Skin	4 (21.1)
Eye	2 (10.5)
Kidney	2 (10.5)
Liver	2 (10.5)
Heart	1 (5.3)

IEI, inborn errors of immunity; GI, gastrointestinal; IQR, interquartile range; BMI, body mass index; IBD, inflammatory bowel disease.

TABLE 3 | Clinical characteristics of 19 patients diagnosed with inborn errors of immunity who required endoscopic evaluations.

No.	Diagnosis	Gene	Sex	Age at GI symptoms onset (yrs)	GI manifestations	Other involved organ	IBD-like colitis	Neoplasms (premalignant & malignant lesions)	Treatment for GI symptoms
1	CVID	N/A	Female	0.5	Diarrhea, Growth retardation	Liver, Kidney, Lung, Heart	–	Colonic tubular adenoma	Steroid
2	CVID	N/A	Female	16.3	Diarrhea, Abdominal pain, Growth retardation	Lung	+ (UC)	–	Mesalazine
3	APDS	PI3K mutation	Female	5.6	Hematochezia	Lung, Lymph node	–	MALT lymphoma	Sirolimus
4	APDS	PI3K mutation	Male	4.2	Hematochezia	Salivary gland	–	–	Sirolimus
5	CGD	CYBB mutation	Male	0.1	Diarrhea, Growth retardation, Perianal abscess	None	+ (CD)	–	Scheduled for HCT
6	CGD	CYBB mutation	Male	9.3	Diarrhea, Hematochezia, Growth retardation, Oral ulcer	Lung, Eye	+ (UC)	–	Mesalazine
7	CGD	CYBB mutation	Male	8.5	Oral ulcer	None	+ (CD)	–	Mesalazine
8	CGD	CYBB mutation	Male	2.5	Diarrhea, Hematochezia	None	+ (CD)	–	Steroid, HCT
9	GSD type Ib	SLC37A4 mutation	Male	2.6	Diarrhea, Growth retardation	None	+ (CD)	–	Mesalazine, HCT
10	CTLA4 deficiency	CTLA4 mutation	Female	18.4	Diarrhea	Lung, Skin	–	Early gastric carcinoma	Subtotal gastrectomy
11	CTLA4 deficiency	CTLA4 mutation	Female	10.0	Diarrhea, Hematochezia,	Lung, Eye, Kidney, Liver, Skin, Salivary gland	+ (UC)	–	Mesalazine, Abatacept
12	IPEX	FOXP3 mutation	Male	8.3	Diarrhea, Hematochezia, Abdominal pain	None	+ (CD)	–	MTX, Adalimumab, HCT
13	XIAP	XIAP mutation	Male	8.4	Diarrhea, Abdominal pain, Growth retardation	Skin	+ (CD)	–	Mesalazine, Adalimumab, Infliximab
14	XLP	SH2D1A mutation	Male	10.7	Incidental finding of malignancy	None	–	–	HCT
15	XLP	SH2D1A mutation	Male	1.2	Hematochezia	None	–	–	HCT
16	Osteopetrosis	CLCN7 mutation	Male	17.5	Diarrhea, Abdominal pain, Perianal fistula	None	+ (UC)	–	Azathioprine, Mesalazine, Infliximab
17	STAT1 GOF	STAT1 mutation	Female	24.8	Abdominal pain	Lung, Skin	–	–	Prokinetics
18	Blau syndrome	NOD2 mutation	Male	0.5	Diarrhea	Skin	+ (CD)	–	Methotrexate, Steroid
19	CRMO	N/A	Male	13.9	Diarrhea, Abdominal pain, Growth retardation	Skin, Bone (costovertebral junction, femur, patella, tibia, talus)	+ (CD)	–	Azathioprine, Mesalazine, Infliximab

GI, gastrointestinal; IBD, inflammatory bowel disease; CVID, common variable immunodeficiency; N/A, non-acting; UC, ulcerative colitis; APDS, activated PI3k delta syndrome; PI3K, phosphorinositide 3 kinase; MALT lymphoma, mucosa associated lymphoid tissue lymphoma; CGD, chronic granulomatous disease; CYBB, cytochrome b-245 β chain; CD, Crohn's disease; HCT, hematopoietic cell transplantation; GSD, glycogen storage disease; SLC, solute carrier; CTLA4, cytotoxic T lymphocyte-associated antigen-4; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked; FOXP3, Forkhead box protein P3; MTX, methotrexate; XIAP, X-linked inhibitor of apoptosis; XLP, X-linked lymphoproliferative syndrome; SH2D1A, SH2 domain containing 1A; CLCN7, chloride voltage-gated channel 7; STAT1, signal transducer and activator of transcription 1; GOF, gain of function; NOD2, nucleotide binding oligomerization domain containing 2; CRMO, Chronic recurrent multifocal osteomyelitis.

chronic recurrent multifocal osteomyelitis, CRMO (**Figure 3B**)]; one patient with defects in intrinsic and innate immunity [osteopetrosis (**Figure 3C**)]; and one patient with predominantly antibody deficiencies (CVID).

One out of four patients with CGD showed colonic leopard sign appearing as brown dots distributed across a yellowish edematous mucosa which means microscopically that aggregation of pigment-laden macrophages on the mucosa (**Figure 4**).

Histopathologic Findings

The histopathologic findings of the study subjects are depicted in **Table 5**. Mucosal ulcer and inflammation were commonly observed endoscopic pathologies in all patients. All CVID patients were found to have intraepithelial lymphocytosis (IEL) of the GI tact, and one patient showed apoptosis and atrophy of duodenal villi (**Figure 5A**). Of note, this patient had severe profuse diarrhea due to a norovirus infection that led to

TABLE 4 | Endoscopic findings in patients with inborn errors of immunity.

	Endoscopic Findings (*n* = 19)	Predominantly antibody deficiencies (*n* = 4)		Congenital defects of phagocyte number or function (*n* =5)		Defects in intrinsic and innate immunity (*n* = 2)		Disease of immune dysregulation (*n* = 6)				Auto-inflammatory Disorders (*n* = 2)	
		CVID	APDS	CGD	GSD type 1b	Osteopetrosis	STAT1 GOF	IPEX	XIAP	CTLA4 deficiency	XLP	Blau syndrome	CRMO
Esophagus	Esophagitis	–	–	1/4[e]	–	–	1/1	–	–	1/2[i]	–	–	–
	Esophageal ulcer	–	–	1/4[e]	–	–	1/1	–	–	1/2[i]	–	–	–
	Esophageal varix	1/2[a]	–	–	–	–	–	–	–	1/2[i]	–	–	–
Stomach	Chronic gastritis	1/2[b]	1/2[c]	1/4[f]	-	1/1	-	-	1/1	2/2[i,j]	1/2[k]	-	-
	Gastric ulcer	1/2[a]	-	-	-	-	-	-	-	1/2[i]	-	1/1	-
	Early gastric cancer	-	-	-	-	-	-	-	-	1/2[i]	-	-	-
Duodenum	Duodenitis	–	–	–	–	–	–	–	1/1	–	–	–	–
	Duodenal ulcer	1/2[a]	–	–	–	–	–	–	1/1	1/2[i]	–	1/1	–
	Lymphoid hyperplasia	1/2[b]	2/2[c,d]	–	–	–	–	–	–	1/2[i]	–	–	–
Ileum	Ileitis	-	1/2[d]	1/4[e]	1/1	1/1	-	-	-	-	-	1/1	-
	Atrophic change	-	-	-	-	-	-	-	-	1/2[i]	-	-	-
	Lymphoid hyperplasia	2/2[a,b]	2/2[c,d]	-	-	-	-	-	1/1	1/2[i]	-	-	-
Colon	Lymphoid hyperplasia	1/2[b]	1/2[c]	1/4[h]	–	–	–	–	–	–	–	–	–
	Mucosal edema	1/2[b]	–	1/4[f]	1/1	–	–	1/1	1/1	1/2[i]	–	–	1/1
	Ulcer	1/2[b]	–	3/4[e,f,g]	1/1	1/1	–	1/1	1/1	1/2[i]	1/2 [k]	–	1/1
	Adenoma	1/2[a]	–	–	–	–	–	–	–	–	–	–	–

**Each alphabet represents one patient.*

CVID, common variable immunodeficiency; APDS, activated PI3k delta syndrome; CGD, chronic granulomatous disease; GSD, glycogen storage disease; STAT1, signal transducer and activator of transcription 1; GOF, gain of function; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked; XIAP, X-linked inhibitor of apoptosis; CTLA4, cytotoxic T lymphocyte-associated antigen 4; XLP, X-linked lymphoproliferative syndrome; CRMO, chronic recurrent multifocal osteomyelitis.

significant weight loss. Likewise, all APDS patients showed IEL in the GI tract. Twelve patients (12/19, 63.2%) were found to have IBD features on biopsies taken from the GI tract. Eight patients had histopathologic features of Crohn's disease such as active inflammation and non-caseating granuloma (**Figure 5B**); they were diagnosed with CGD, GSD type 1b, IPEX, XIAP, Blau syndrome and CRMO. Four patients (CVID, APDS, CGD, and Osteopetrosis) had features of ulcerative colitis such as crypt distortion and cryptitis/crypt abscess (**Figure 5C**).

DISCUSSION

Few studies analyzing the GI tract diseases in IEI have been conducted to date. In addition, there is little data on endoscopic and histopathologic findings of the GI tract in IEI patients (9–13). With increasing awareness, IEI is an emerging disease in Asia, however, there are scarce data on IEI including South Korea (14). This is the first study to observe the clinical, endoscopic, and histopathologic findings of the GI tract in IEI patients in a single center over the long term.

Depending on the nature of the specific type of IEI, the frequency of GI manifestations can range from 5 to 50% (6). In our cohort, 33.3% of patients (55/165) had GI manifestations, and 11.5% of all IEI patients (19/165) had severe GI manifestations that required endoscopy. These frequencies are consistent with previously reported studies (6). In our subjects, diarrhea was the most common symptom (13/19, 68.4%), followed by hematochezia, abdominal pain and growth impairment, which were previously reported as prevalent GI symptoms in IEI patients (7, 9, 15–22). It is already known that growth impairment was frequently detected, and the risk of growth failure is four times higher than in the healthy pediatric population (23–25). Growth can be affected in a variety of ways in patients with IEI: hyper catabolic states such as recurrent inflammatory and/or infectious conditions; reduced caloric intake because of low ingestion or malabsorption; endocrine disorders; osteoarticular dysplasia; and genetic syndromes such as Kabuki syndrome (26). In the studies regarding to patients with IEI, growth impairment was found in 11–28% of patients (27, 28). In this study, the proportion of patients with growth impairment was higher than in previous studies (6/19, 31.6%), which may be due to a cohort of patients with severe GI symptoms requiring endoscopies.

Malignancy is the second most prevalent cause of mortality in patients with IEI, after infection (29). The lifetime risk of cancer in children with IEI is estimated to be 5–25% (30, 31), and the relative risk of cancer in patients with IEI is 1.4 to 1.6 times greater than that in the general population (32, 33). It has been reported that genomic instability due to defective DNA repair processes, lack of control of chronic inflammation, susceptibility to oncogenic viruses (such as human papillomavirus or Epstein-Barr virus) and other unknown mechanisms increase the risk of malignancies (29, 34, 35).

The GI tract malignancy may be diagnosed at initial diagnosis of IEI or during a follow-up visit in a patient with known IEI. Within the USIDNET cohort, 171 different cancers were reported, of which GI cancer accounted for 8%. In our cohort,

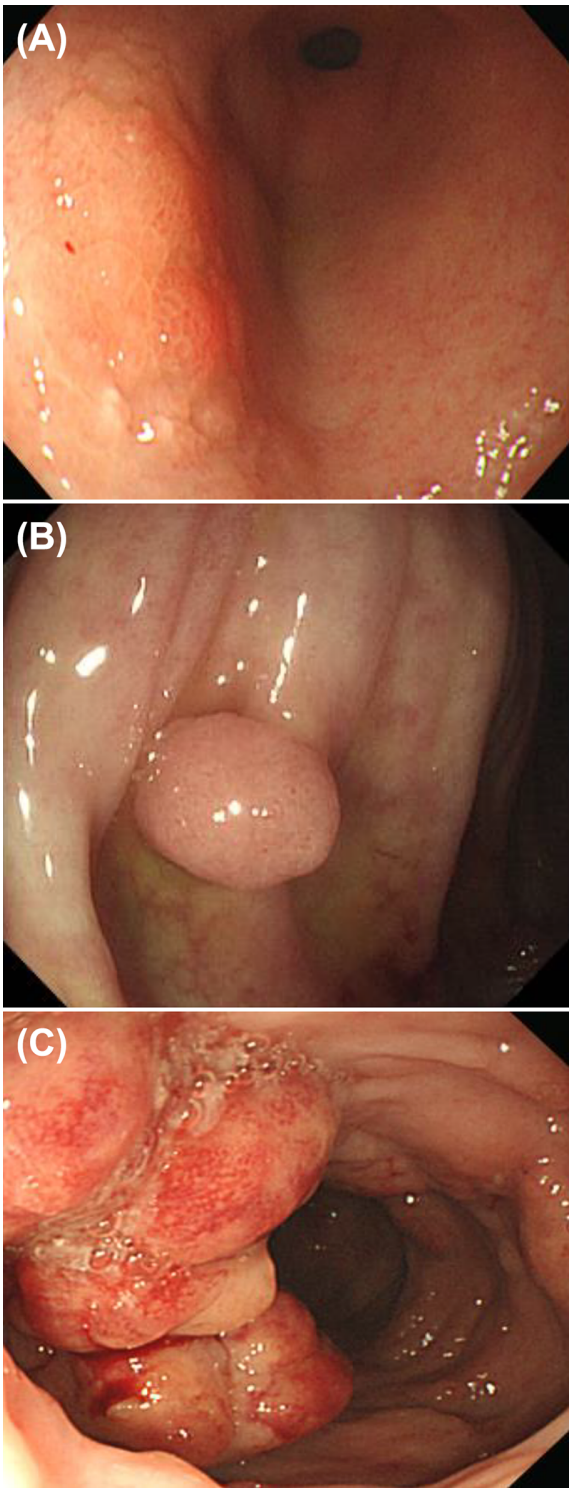

FIGURE 2 | Gastrointestinal tract malignancy in patients with primary immunodeficiency **(A)** Early gastric carcinoma in a patient with CTLA4 deficiency. **(B)** Colonic tubular adenoma in a patient with common variable antibody deficiency. **(C)** Colonic MALT lymphoma in a patient with activated PI3Kδ syndrome.

1.8% of patients with IEI (3/165) were diagnosed with GI tract neoplasms (premalignant and malignant lesions) during the study period: early gastric carcinoma in CTLA 4 deficiency, MALT lymphoma in APDS, and colonic tubular adenoma in CVID. However, in patients who underwent endoscopic evaluation for severe GI symptoms, the rate of GI tract neoplasms increased up to 15.8% (3/19).

Currently, there are no specific recommendations for cancer screening in high-risk IEI individuals, and the prerequisites listed in screening protocols differ from country to country because of variations in cancer incidence, mortality rates, racial differences, and socio-economic states. In case of one of patient with APDS who developed MALT lymphoma, the patient had severe hematochezia required initial colonoscopy which showed only adenomatous changes in the colon at 6 years old. Therefore, colonoscopy was performed regularly thereafter. Five years later (11 years old), multi-lobulated masses were observed in the colon which revealed MALT lymphoma for which careful observation was continued. Later, the patient's genetic study revealed the diagnosis of APDS and sirolimus was started. MALT lymphomatous lesions disappeared six months after the initiation of sirolimus. In a CTLA 4 deficiency patient, gastric adenoma was diagnosed at the age of 19 and progressed to gastric adenocarcinoma six years later which required surgical resection. Clinicians should make sure not to miss routine assessments that can identify the cause of GI symptoms such as cancer, and radiologists and pathologists should be aware of the manifestations of IEI when interpreting test results. In addition, it is important to ensure that patients and their parents are aware of the possibility of malignancies and that they know to report any changes in their health status to their physicians.

With the recent advances in diagnostic and therapeutic strategies for IEI, the lifespan of patients is increasing at a remarkable rate, and inflammatory and autoimmune manifestations are emerging as an important issue. Immune dysregulation in IEI leads to autoimmune manifestations by affecting variable immune pathways such as T cell development/tolerance, T cell signaling, interferon signaling pathway and/or resolution of inflammatory (36). In addition, the influence of chronic and recurrent infection through suboptimal and chronic immune response, bystander activation, super antigens activation and molecular mimicry is thought to play an important role in the development of autoimmunity (37). For example, Broides et al. reported that non-infectious colitis is common, and elevation of fecal calprotectin, which is a quantitative measure of intestinal inflammation and indicates the presence of inflammation in the GI tract, is common in asymptomatic CGD patients without overt GI manifestations (38).

In the same vein, IEI is a rare but important cause of IBD, especially in very early onset IBD (39). IBD is a multifactorial idiopathic chronic inflammatory disease of the GI tract caused by a dysregulated immune response to host intestinal microbiota (40, 41), and may be the first or only symptom of underlying IEI. IBD-like colitis in IEI can be further understood through the lens of IEI-associated autoimmunity to an idiopathic autoimmune and inflammatory condition. As described above, 63.2% (12/19) of patients in our cohort also showed inflammatory and

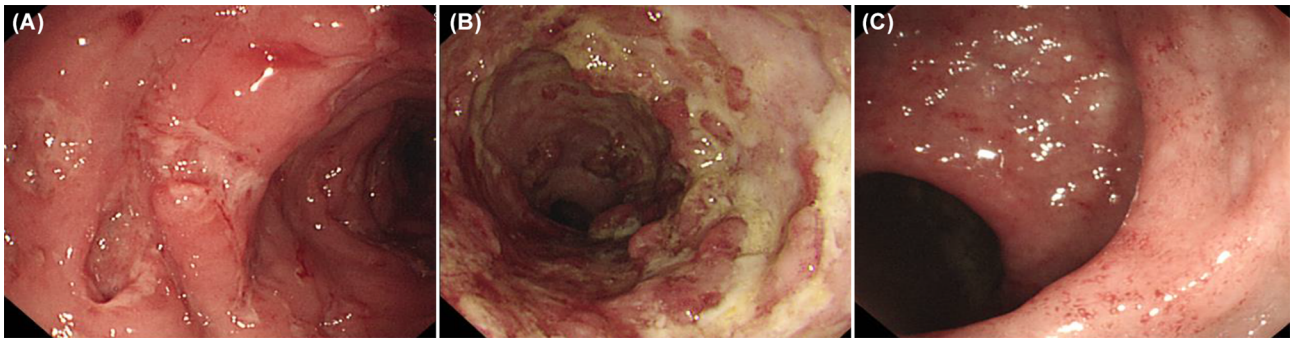

FIGURE 3 | Inflammatory bowel disease like colitis in patients with primary immunodeficiency. **(A)** Multiple large deep ulcers on whole colon in a patient with X-linked inhibitor of apoptosis protein deficiency. **(B)** Longitudinal large deep ulcer on whole colon in a patient with Chronic recurrent multifocal osteomyelitis. **(C)** Mucosal edema, tiny superficial ulcer, loss of vascularity on colon in patients with osteopetrosis.

autoimmune features in the GI tract. These patients showed endoscopic and histopathologic features of IBD and were treated according to the IBD guideline (8). If IEI-associated IBD is suspected, early immunologic tests should be performed. The types of IEI related to IBD are consistent with genetic defects underlying IEI that are known to be associated with autoimmunity: CGD, X-linked proliferative disease, IPEX, CTLA4 deficiency, and CVID (36).

The diagnosis of underlying IEI has an important impact on the therapeutic decisions that are made in cases of IBD. Some treatments for certain types of IEI have been found to be effective against IBD. For example, abatacept is effective in patients with CTLA4 deficiency, as seen in a patient in our study, and HCT is known as a therapeutic option for treatment of XIAP, CGD, and IL-10 deficiency and associated IBD-like colitis. Early diagnosis and treatment of IEI-associated IBD is critical to improving disease outcomes and preventing and mitigating severe complications. Endoscopy may be helpful in diagnosing IEI-induced GI manifestations, assessing the severity of the disease, and monitoring the response to medical treatment (9). The GI tracts of patients with IEI shows a broad spectrum of histopathologic patterns. The condition can mimic lymphocytic gastroenteritis, granulomatous disease, acute graft-versus-host disease (GVHD), and IBD (2). The histopathologic findings in our study showed that features of GVHD as apoptosis and prominent lymphocytosis, features of celiac disease as villous blunting with IEL, and features of IBD as cryptitis, crypt distortion or non-caseating granuloma. From this point of view, pathologists should remember that certain features of IBD may appear in IEI patients. Although the characteristic feature of IEI is increased susceptibility to infection, many cases are associated with GI disease and initially present with GI symptoms, meaning that a routine evaluation of the GI tract is necessary. A history of recurrent infections, atypical clinical and/or histologic features of an uncommon pattern of GI disease, or a poor response to conventional therapy should facilitate further immunologic evaluation.

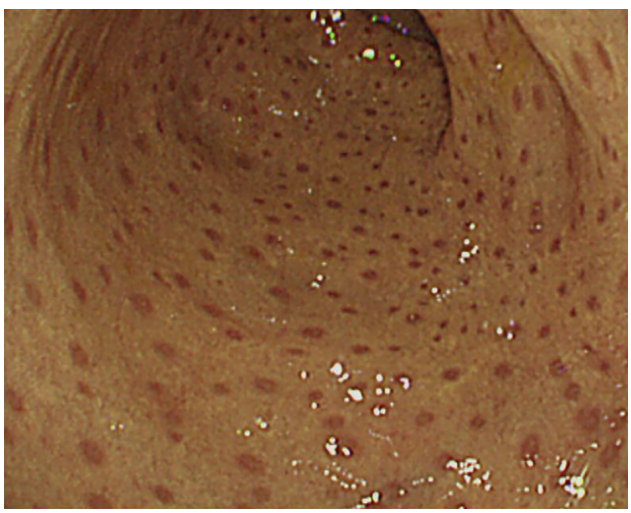

FIGURE 4 | Colonoscopic 'leopard sign' in Chronic granulomatous disease. Endoscopic view of the colonic mucosae showing the leopard sign appearing as brown dots distributed across a yellowish edematous mucosa.

In conclusion, GI manifestations may appear concurrently with the onset of IEI or occur during the course of IEI. In addition, it is necessary to always consider that autoimmune/inflammatory diseases and malignancies may occur in patients who have already been diagnosed with IEI. An investigation of immunodeficiency in patients with atypical GI symptoms can lead to a correct diagnosis and appropriate disease-specific therapy. Thus, there is a need for increased awareness of GI manifestations in IEI. Gastroenterologists and immunologists should consider endoscopy when atypical and/or refractory GI manifestations appear in IEI patients. A collaborative approach among gastroenterologists and immunologists in evaluating IEI patients with refractory GI symptoms is required to better understand the full spectrum of GI tract diseases and associated complications. Also, when physicians in various fields, such as

TABLE 5 | Histopathologic findings of GI tract in patients with inborn errors of immunity.

Histopathologic Findings (*n* = 19)		Predominantly antibody deficiencies (*n* = 4)		Congenital defects of phagocyte number or function (*n* =5)		Defects in intrinsic and innate immunity (*n* = 2)		Disease of immune dysregulation (*n* = 6)				Autoinflammatory Disorders (*n* = 2)	
		CVID	APDS	CGD	GSD type 1b	Osteopetrosis	STAT1 GOF	IPEX	XIAP	CTLA4 deficiency	XLP	Blau syndrome	CRMO
Esophagus	Esophagitis	–	–	1/4[e]	N/A	–	–	–	–	1/2[h]	–	–	–
	Esophageal ulcer	–	–	1/4[e]		–	1/1	–	–	–	–	–	–
	Eosinophilic infiltration	1/2[a]	–	–		–	–	–	–	–	–	–	–
Stomach	H.pylori (-) gastritis	1/2[b]	1/2[c]	1/3[f]	N/A	1/1	-	-	1/1	2/2[h,i]	1/2 [j]	1/1	-
	H.pylori (+) gastritis	-	1/2[d]	-		-	-	-	-	-	-	-	-
	Intestinal metaplasia	-	-	-		-	-	-	-	1/2[h]	-	-	-
	Tubular adenocarcinoma	-	-	-		-	-	-	-	1/2 [h]	-	-	-
Duodenum	Duodenitis	–	1/2[d]	–	N/A	–	–	–	1/1	–	–	1/1	–
	Apoptosis	1/2[a]	–	–		–	–	–	–	–	–	–	–
	Atrophy of villi	1/2[a]	–	–		–	–	–	–	–	–	–	–
	Intraepithelial lymphocytosis	1/2[a]	–	–		–	–	–	–	–	–	–	–
Ileum	Ileitis	-	-	1/1	1/1	-	-	-	-	-	-	1/1	1/1
	Intraepithelial lymphocytosis	1/2[a]	2/2[c,d]	-	-	-	-	-	-	-	-	-	-
	Lymphoid hyperplasia	-	2/2[c,d]	-	-	-	-	-	-	1/2[i]	-	-	-
	Eosinophilic infiltration	-	1/2[d]	-	-	-	-	-	-	1/2[h]	-	-	-
Colon	Active inflammation	1/2[b]	1/2[c]	3/3[e,f,g]	1/1	–	–	1/1	1/1	1/2[i]	1/2 [j]	1/1	1/1
	Apoptosis	1/2[b]	–	–	–	–	–	–	–	–	–	–	–
	Pigmented macrophage	–	–	1/3[g]	–	–	–	–	–	–	–	–	–
	Crypt distortion	–	–	1/3[f]	–	1/1	–	–	–	–	–	–	–
	Cryptitis/Crypt abscess	1/2[b]	–	1/3[f]	–	1/1	–	–	–	1/2[i]	–	–	1/1
	Non-caseating granuloma	–	–	1/3[f]	1/1	–	–	1/1	1/1	–	–	–	–
	Intraepithelial lymphocytosis	1/2[b]	2/2[c,d]	1/3[f]	–	–	–	–	–	1/2[i]	–	–	–
	Eosinophilic infiltration	–	1/2[d]	–	–	–	–	–	–	1/2[i]	–	–	–
	Tubular adenoma	1/2[a]	–	–	–	–	–	–	–	–		–	–
	MALT lymphoma	–	1/2[c]			–	–	–	–	–		–	–

**Each alphabet represents one patient.*

CVID, common variable immunodeficiency; APDS, activated PI3k δ syndrome; CGD, chronic granulomatous disease; GSD, glycogen storage disease; STAT1, signal transducer and activator of transcription 1; GOF, gain of function; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked; XIAP, X-linked inhibitor of apoptosis; CTLA4, cytotoxic T lymphocyte-associated antigen-4; XLP, X-linked lymphoproliferative syndrome; CRMO, chronic recurrent multifocal osteomyelitis; MALT lymphoma, mucosa associated lymphoid tissue lymphoma.

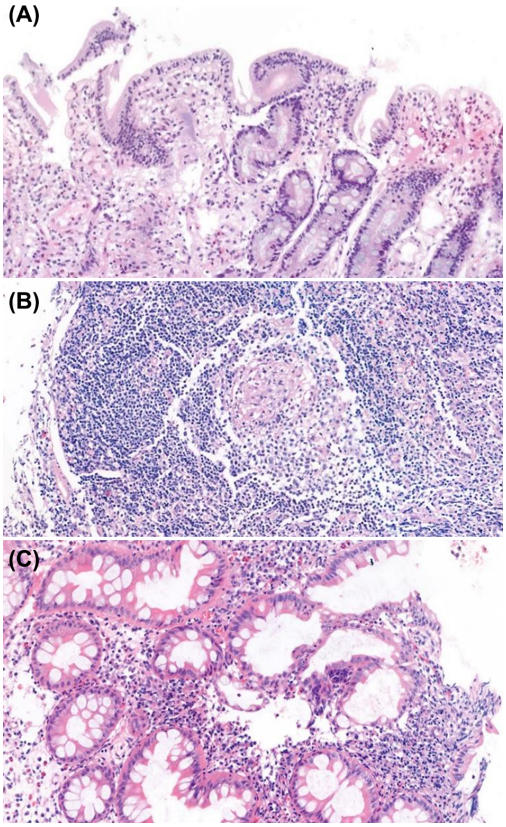

FIGURE 5 | Histopathologic findings of endoscopic biopsies (H&E staining). **(A)** Duodenal biopsy with atrophy of villi, apoptotic bodies and increased intraepithelial lymphocytes, consistent with common variable immunodeficiency. **(B)** Colonic biopsy with non-caseating granuloma in patient with X-linked inhibitor of apoptosis protein syndrome. **(C)** Colonic biopsy with cryptitis and crypt distortion in patient with osteopetrosis.

radiologists and pathologists, increase their awareness of IEI-specific complications, early diagnosis, and disease-specific treatment for IEI can be made more appropriately.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Samsung Medical Center (IRB File No. 2021-01-067). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Guarantor of the article: ESK. ESK contributed in the conception and design of the study, acquisition, analysis and interpretation of data, drafting of the initial manuscript, and critical revision for important intellectual content. DK, YY, YK and SP contributed to the acquisition, analysis and interpretation of data. JK, KMA and SA contributed to the acquisition and interpretation of data. YHC contributed to the conception and design of the study and critical revision for important intellectual content. Y-JK contributed to the conception and design of the study, analysis and interpretation of data, drafting of the initial manuscript and critical revision for important intellectual content. MJK contributed to the conception and design of the study, interpretation of data, drafting of the initial manuscript, and critical revision for important intellectual content. All authors contributed to the article and approved the submitted version

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Clinical Application of Metagenomic Next-Generation Sequencing for Suspected Infections in Patients With Primary Immunodeficiency Disease

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Background: Infections are the major cause of morbidity and mortality in patients with primary immunodeficiency disease (PID). Timely and accurate microbiological diagnosis is particularly important in these patients. Metagenomic next-generation sequencing (mNGS) has been used for pathogen detection recently. However, few reports describe the use of mNGS for pathogen identification in patients with PID.

Objective: To evaluate the utility of mNGS for detecting pathogens in patients with PID, and to compare it with conventional microbiological tests (CMT).

Methods: This single center retrospective study investigated the diagnostic performance of mNGS for pathogens detection in PID patients and compared it with CMT. Sixteen PID patients with suspected infection were enrolled, and medical records were analyzed to extract detailed clinical characteristics such as gene variation, immune status, microbial distribution, time-consuming of mNGS and CMT, treatment, and outcomes.

Results: mNGS identified pathogenic microbe in 93.75% samples, compared to 31.25% for culture and 68.75% for conventional methods, and detected an extra 18 pathogenic microorganisms including rare opportunistic pathogens and *Mycobacterium tuberculosis*. Pathogen identification by mNGS required 48 hours, compared with bacterial culture for 3-7 days and even longer for fungus and *Mycobacterium tuberculosis* culture.

Conclusions: mNGS has marked advantages over conventional methods for pathogenic diagnosis, particularly opportunistic pathogens and mixed infections, in patients with PID. This method might enable clinicians to make more timely and targeted therapeutic decisions, thereby improving the prognosis of these patients.

Keywords: metagenomic next-generation sequencing, infection, diagnosis, primary immunodeficiency disease, conventional microbiological tests

INTRODUCTION

Primary immunodeficiency disease (PID) or inborn errors of immunity are caused by monogenic mutations, resulting in loss- or gain-of-function of the encoded protein. They manifest as increased susceptibility to infectious diseases, as well as a growing diversity of autoimmune, autoinflammatory, allergic, lymphoproliferative, and/or malignant phenotypes. They comprise 404 distinct disorders, with 430 different gene defects (1, 2). Infection is a major cause of repeated hospitalization and eventual death in children with PID. A report from France shows that 85% of non-transplant PID patients were admitted to hospital related to acute infections (3). Timely and effective anti-infection therapy is of great importance for reducing infection mortality and improving transplant success rates in PID patients.

Metagenomic next-generation sequencing (mNGS) has been used in many fields in recent years; this technology allows simultaneous and independent sequencing of thousands to billions of DNA fragments, thereby facilitating an unbiased approach to broad identification of both known and unexpected pathogens (or even the discovery of new organisms). Compared with culture-based methods, mNGS offers advantages such as short turn-around times and unbiased quantitative or semi-quantitative analysis. In addition, multiple agents across the full microbial spectrum can be detected simultaneously by mNGS, along with identification of non-culturable microbes (4). The cost of mNGS has fallen by several orders of magnitude since its advent in 2004; it has emerged as an enabling technological platform for detection of microorganisms in clinical samples. Recent studies used mNGS to identify pathogens in the respiratory and central nervous systems, and pathogens that cause focal and bloodstream infections, and pathogens in immunosuppressed patients (5–14). However, only a few studies report the use of mNGS to detect infectious agents in patients with PID (15–17). Due to the unique characteristics and overall severity of infections in these patients, rapid and accurate diagnostic methods are needed urgently. This study aimed to determine whether mNGS technology can meet this need by assessing its ability to detect pathogens in patients with PID, and by comparing its efficacy with that of conventional microbiological tests (CMT).

MATERIALS AND METHODS

Study Design and Participants

This study comprised a retrospective analysis of data from 16 PID patients with suspected infections admitted to Children's Hospital of Chongqing Medical University in Chongqing, China from October 2018 to December 2020. Samples from these patients were subjected to both CMT and mNGS. Extensive phenotype information, including clinical and laboratory data, were available for all patients to facilitate interpretation of results. Infections were suspected in patients presenting with symptoms such as fever, cough, weakness, headache, hemiplegia, abdominal pain, and ostealgia. The study was approved by the Medical Ethics Committee of the Children's Hospital of Chongqing Medical University and was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All patients provided written informed consent prior to sample collection.

Specimen Collection and Processing

During enrollment, samples from all subjects underwent CMT ordered by their treating clinicians. Different clinical specimens were collected for testing based on the type of suspected infection (i.e., cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BALF), peripheral blood, sputum, liver biopsy tissue, pus, and bone biopsy tissue). The CMT included blood/sputum/BALF/CSF cultures, serological tests, molecular diagnostic tests, and antigen detection. CMT were conducted in accordance with a clinical assessment of necessity. At the same time, additional samples were collected and transported overnight to Kindstar Global Laboratories (Wuhan, China) for mNGS.

Sample Processing and Library Construction

Before nucleic acid extraction, samples were processed as follows: tissue samples were ground into a homogenate, and sputum was liquefied. DNA was extracted with TIANamp Micro DNA Kit (TIANGEN BIOTECH, Beijing, China) from collected samples according to the manufacturer's instructions. Next, libraries were constructed for NGS, as described previously (13). When patients underwent mNGS, due to sample volume, test price and other factors, no relevant information about RNA viruses was collected from all patients included in this study.

Next Generation Sequencing

Samples were transported to Kindstar Global Wuhan for PMseqTM library construction and Illumina MiniSeq platform or Illumina NextSeq 550 platform for high-throughput metagenomic sequencing. The minimum limit of microbe detection of this technique is 100 copies/mL (viruses = 1000 copies/mL). The specificity and repeatability of microbial detection were both > 99% when the copy number was above the minimum limit of detection.

Analysis of Sequencing Data

As described previously, adapter contamination, low quality and low-complexity reads were quality filtered using an in-house program (13). Next, Burrows-Wheeler Alignment (Version: 0.7.10) was used to map the filtered sequences to a human reference database that includes hg38 and the Yanhuang genome sequence. The Remaining data were classified into four Microbial Genome Databases: viruses, bacteria, fungi, and parasites. Classification reference databases were downloaded from NCBI (ftp://ncbi.nlm.nih.gov/genomes/). The depth and coverage of each species were calculated by Soap Coverage software from the SOAP website (http://soap.genomics.org.cn/). Next, the parameter values were normalized according to data size and detected species listed in the suspected background database

were filtered, as previously reported (13, 18). Based on the relative abundance of microbes detected by NGS in healthy control samples, we set up the threshold for each microbe to allow further validation. Pathogens with the highest absolute abundance within their genus, and pathogens ranked in the top 10 fungi, viruses, and parasites, and ranked in the top 50 bacteria (in terms of relative abundance after the previous two screening steps), were selected. With respect to intracellular bacteria (i.e., *Mycobacterium tuberculosis* and *brucella*) and some fungi (i.e., *cryptococcus*), as long as the test data are compared with the above reference genomes, it is necessary to consider whether they are infectious pathogens in combination with clinical practice. If detected pathogens were common infectious pathogens, they were considered to be causative agents. In the case of uncommon pathogens, the mNGS results were interpreted in the context of the patient's clinical features; otherwise, the detected reads were classified as "non-pathogenic" microbe sequences. Strictly map reads number (SMRN) and genomic coverage were analyzed. SMRN represents the number of sequences that are strictly aligned with the microorganism (genus/species), which can reflect the pathogenicity of detected pathogens to some extent. SMRN are affected by content of pathogen in the sample, the size of pathogen genome, the amount of nucleic acid extracted from the sample, and other factors. High SRMN in an detected pathogen does not completely mean it is pathogenic and vice versa. Genomic coverage refers to the percentage of the nucleic acid sequence length of the microorganism detected to the genome sequence length of the microorganism. Generally speaking, the higher the genomic coverage, the higher the credibility of the pathogen detected. But it also influenced by the type of pathogen.

Statistical Analysis

Due to the small number of patients enrolled, differences among groups were analyzed using a two-tailed independent-samples t test. A P value <0.05 was considered significant. Data analysis was performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Basic Clinical Information

Sixteen patients with PID (age range, 4 months to 16 years) were enrolled. Specific primary diseases were as follows: X-linked-hyper IgM syndrome (XHIM) caused by a *CD40LG* mutation (n=4, P1 to P4); Wiskott-Aldrich syndrome (WAS) caused by a *WASP* mutation (n=2, P5 and P6); CTLA4 deficiency(P7); PSTPIP1-associated myeloid-related proteinemia inflammatory syndrome (PAMI) caused by a *PSTPIP1* mutation (P8); X-linked lymphoproliferative disease (XLP) caused by a *SH2D1A* mutation (P9); NEMO deficiency caused by a *IKBKG* mutation (P10); chronic granulomatous disease (CGD) caused by a *CYBB* mutation (n=2, P11 and P12); Artemis deficiency caused by a *DCLRE1C* mutation (P13); Mendelian susceptibility to mycobacterial disease (MSMD) caused by a *STAT1* AD loss-of-function mutation (P14); X-linked agammaglobulinemia (XLA) caused by a *BTK* mutation (P15); and activated phosphoinositide 3-kinase-δ syndrome (APDS) caused by a *PIK3CD* AD GOF mutation (P16). The basic information, including age, gender, infection data, immunological characteristics, and gene variation are shown in **Supplementary Table 1**.

The clinical presentations of patients at the time of sample collection were as follows: patient P1 had claudication of the left lower extremity, weakness of the left hand, and intermittent fever; P2 presented mainly with intentional tremor of the right upper limb, unsteady gait, and occasional fever; P3 suffered dizziness, headache, vomiting, drowsiness, slurred speech, unsteady gait, and fever; P15 presented mainly with unstable gait, choking, and intermittent fever; and P16 presented mainly with fever and headache. The above five patients were suspected to have central nervous system (CNS) infections; therefore, cerebrospinal fluid (CSF) was collected and cranial images taken (**Figure 1**). Seven patients (P4, P7 to P11, and P13) all had cough and fever and so were suspected to have pneumonia; therefore, bronchoalveolar lavage fluid (BALF) or sputum was collected. P5 presented with fever and rash; we suspected sepsis and so obtained peripheral venous blood samples. P6 had

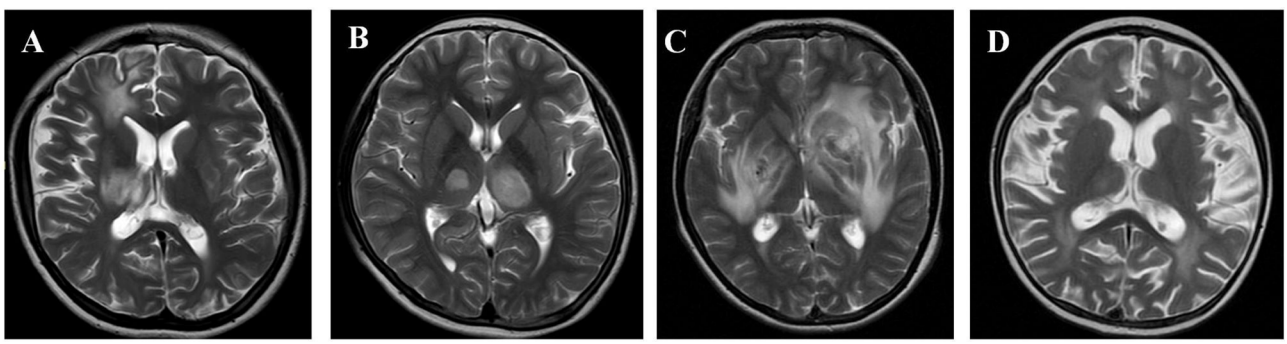

FIGURE 1 | Cranial magnetic resonance imaging of patients. **(A)** Patient P1: Multiple abnormal bilateral signals in the brain. Both old and new lesions coexist. Demyelinating lesions may be present. **(B)** Patient P2: Sparse abnormal bilateral signals in the brain parenchyma, suggestive of inflammatory lesions. **(C)** Patient P3: Abnormal signals in the brain and cerebellum, particularly in the bilateral basal ganglia and the left frontal lobe, suggestive of infective lesions. **(D)** Patient P15: Abnormal, bilateral symmetrical signals in the white matter, brain atrophy, and similar signals in the thalamus.

abdominal pain and liver space-occupying lesions; liver biopsy tissues were collected and checked for specific pathogen infection or tumors. In patient P12, abdominal pain and fever were the main symptoms; abdominal ultrasound indicated that the main lesion was in the parenchymal components of the right lobe of the liver. The patient's underlying disease was CGD. Therefore, liver abscess was considered and he received imipenem cilastatin and linezolid. After abdominal ultrasound showed fluid lesions, the liver abscess was punctured, drainage was performed, and the pus was collected. In patient P14, the main manifestations were abnormal gait, which progressed gradually to ostealgia in the head, chest, waist, and lower limbs, accompanied by intermittent fever. CT revealed multiple areas of bone destruction throughout the body, including the left humerus, left ulnar radius, right femur, right tibia, left fibula, frontal bone, parietal bone, occipital bone, and vertebral bodies C1–2, L1–3, and S1. Considering his primary disease, we suspected infection with pathogens such as *Mycobacterium*. A bone biopsy was performed and bone biopsy tissue was collected. The suspected infections and samples collected are shown in **Figure 2**.

Diagnostic Performance of mNGS

The mNGS sequencing results for 15/16 (93.75%) patients were positive for microbial pathogens (**Table 1**). A comparison of the diagnostic results from mNGS with CMT is shown in **Table 2**. CMT and mNGS results were concordant for 11 of 15 patients (73.3%). However, agreement between the culture method and mNGS was only 33.3%. Positive agreement between mNGS and clinical diagnosis was significantly higher than that of culture and CMT (93.75% *vs.* 31.25% and 68.75%, respectively). In addition, mNGS detected 18 more pathogens than conventional methods in these patients.

Distribution of Identified Pathogens

In the present study, the diagnosis of 15 patients was confirmed by both clinical and microbiological criteria. According to the results, bacteria (73.33%) were the most common pathogens identified, followed by virus (60%), fungi (26.67%), and parasites (13.33%). It is noteworthy that among the 15 patients with identified pathogens, 11 were diagnosed with polymicrobial infections (73.33%). In particular, Cytomegalovirus (CMV) and Epstein-Barr virus (4/15) were the most common pathogens, followed by *Stenomonas maltophilia* (3/15) (**Figure 3**).

The following pathogens were identified in different types of PID patients. Among the four patients with XHIM, two patients had *JC polyomavirus* infection, one patient had toxoplasmosis infection, and one had a mixed infection by *Stenotrophomonas maltophilia, Pneumocystis jirovecii*, and CMV. Both patients with WAS were infected with human herpesviruses, one with EBV and another with CMV. Mixed bacterial infections (including opportunistic pathogens such as *Sphingomonas paucimobilis* and *Stenomonas maltophilia*) were identified in both patients with CGD. Others included *Mycobacterium* (in a patient with APDS), and *Nocardia farcinica* and *Mycobacterium avium* mixed infection (in a patient with MSMD) (**Table 1**).

Comparison of mNGS With CMT

In CSF, BALF, and blood samples, mNGS showed higher sensitivity for microbes detection than CMT; however, there was no difference with respect to sputum, tissue, and pus (**Figure 4**). In samples from 11 patients with polymicrobial infections, multiple microbiological tests were performed to obtain a final pathogenic diagnosis; these were more time-consuming and costly than tests used for monomicrobial infections. However, mNGS yielded consistent results from only one test in 11 of the 15 subjects. We analyzed the time taken to determine the pathogenic diagnosis. For monomicrobial infections, the time for mNGS and CMT was not statistically different; however, for polymicrobial infections, mNGS required significantly less time to identify the pathogens than CMT ($P < 0.05$). In terms of testing time, CMT for the 11 patients took 3–7 days, while the sequencing time was only 48 hours. In terms of detection efficiency, five of the 16 patients were negative by CMT, whereas mNGS detected pathogens in 15 patients; some of these had a mixed infection, suggesting that mNGS has better detection efficiency.

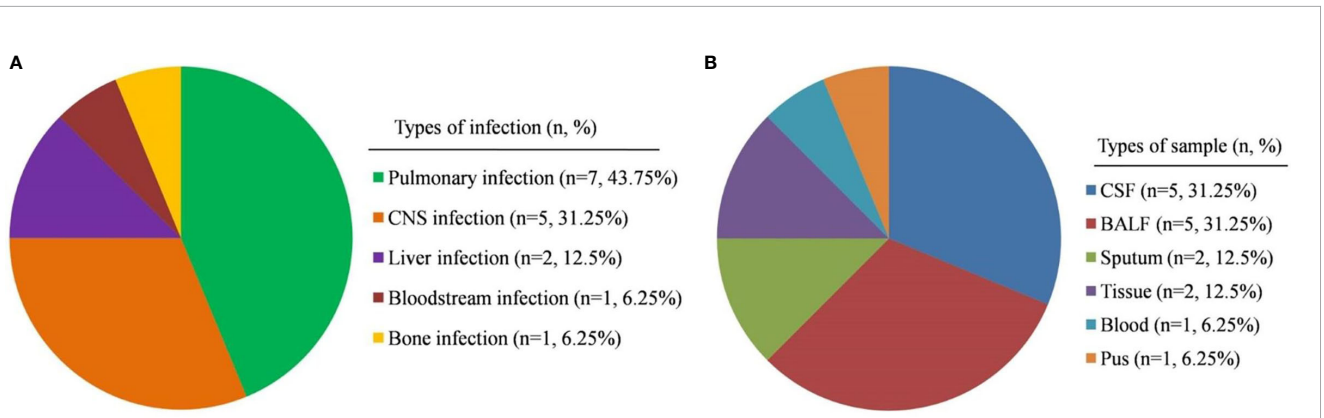

FIGURE 2 | Composition of patients and samples types. **(A)** Type of suspected infection in the PID patients included in this study. **(B)** Types of sample collected in this study. CNS, central nervous system; CSF, cerebrospinal fluid; BALF, bronchoalveolar lavage fluid.

TABLE 1 | Results of CMT and mNGS of various samples for patients with PID.

Patients	Specimen for mNGS	Culture results	Other microbiological testing results	mNGS results (pathogenic)	SMRN	Genomic coverage (%)
P1	CSF	Negative (CSF and blood)	Negative	*JC polyomavirus*	1526	97.563
				BK Virus	20	30.063
				Acidovorax temperans	3301	11.166
				Comamonas testosteroni	591	1.453
				Malassezia globosa	119	0.168
				Acanthamoeba triangularis	890	0.241
P2	CSF	Negative (CSF and blood)	Negative	*JC polyomavirus*	5	14.6
				Acidovorax temperans	30	0.119
				Ralstonia mannitolilytica	18	0.053
P3	CSF	Negative (CSF and blood)	Negative	*Toxoplasma gondii*	151	0.01
P4	sputum	Negative (sputum and blood)	CMV-DNA (+)	*Acinetobacter junii*	567	1.44
				Stenotrophomonas maltophilia	118	0.21
				Pneumocystis jirovecii	2	0.0018
				Cytomegalovirus	2	0.064
P5	blood	Negative (blood)	CMV-DNA (+)	*Cytomegalovirus*	18	1.139
P6	Liver tissue	Negative (blood)	EBV-DNA(+)	*Epstein-barr virus*	18	0.757
P7	BALF	*Klebsiella pneumoniae* (BALF)	Negative	*Klebsiella pneumoniae*	18957	3.0
P8	BALF	Negative (BALF and blood)	Mycoplasma pneumoniae (+)	*Epstein-barr virus*	45	2.781
				Mycoplasman pneumonia	154	2.479
P9	BALF	*Streptococcus pneumoniae*	Negative	*Streptococcus pneumonia*	67	0.434
		Pseudomonas aeruginosa		*Pseudomonas aeruginosa*	6	0.014
		(BALF)		*Haemophilus influenza*	3	0.024
				Epstein-barr virus	2	0.159
P10	BALF	*Klebsiella aerogenes*	Negative	*Stenotrophomonas maltophilia*	364	1.221
		Candida albicans		*Klebsiella aerogenes*	156	0.444
		(BALF)		*Candida albicans*	3380	Copies/ml
				Cytomegalovirus	61	3.761
P11	BALF	Negative (BALF and blood)	Negative	*Haemophilus parainfluenzae*	6	0.100
				Sphingomonas paucimobilis	2	0.007
				Escherichia coli	2	0.005
P12	Pus	*Staphylococcus aureus* (Pus)	Negative	*Stenomonas maltophilia*	19	0.03
				Staphylococcus aureus	5	0.02
P13	sputum	Multiple drug resistant pseudomonas aeruginosa (Sputum)	Negative	*Pseudomonas aeruginosa*	1599	3.572
				Torque teno virus	56	13.687
				Epstein-barr virus	376	10.228
				Cytomegalovirus	23	1.378
P14	Bone biopsy tissue	Negative	PPD (+)	*Nocardia farcinica*	752	0.73
		(Blood)	T-SPOT (+)	*Mycobacterium avium*	2	0.01
P15	CSF	Negative (CSF and blood)	Negative	Negative	/	/
P16	CSF	Negative	Sputum X-pert (+)	*Mycobacterium*	2	0.01
		(CSF and blood)		*Rasamsonia emersonii*	1	0.01

CMV, Cytomegalovirus; EBV, Epstein-barr virus; SMRN, strictly map reads number.

TABLE 2 | Comparison of positive results and agreement among mNGS, CMT and culture method in patients.

Group	mNGS-positive	mNGS-negative	Total number
CMT-positive	11	0	11
CMT-negative	4	1	5
Culture-positive	5	0	5
Culture-negative	10	1	11
Total number	15	1	16

Treatment and Follow-Up

Of the 16 patients, P3 improved significantly after treatment with sulfamethoxazole. P4 improved after treatment with sulfamethoxazole and ganciclovir; P5 and P7–P11 were treated successfully with anti-infection therapy, including antibiotics and antifungal agents. P12 was treated successfully with sulfamethoxazole, imipenem cilastatin, and linezolid. P14 improved after treatment with isoniazid, rifampicin, pyrazinamide, ethambutol, and sulfamethoxazole.

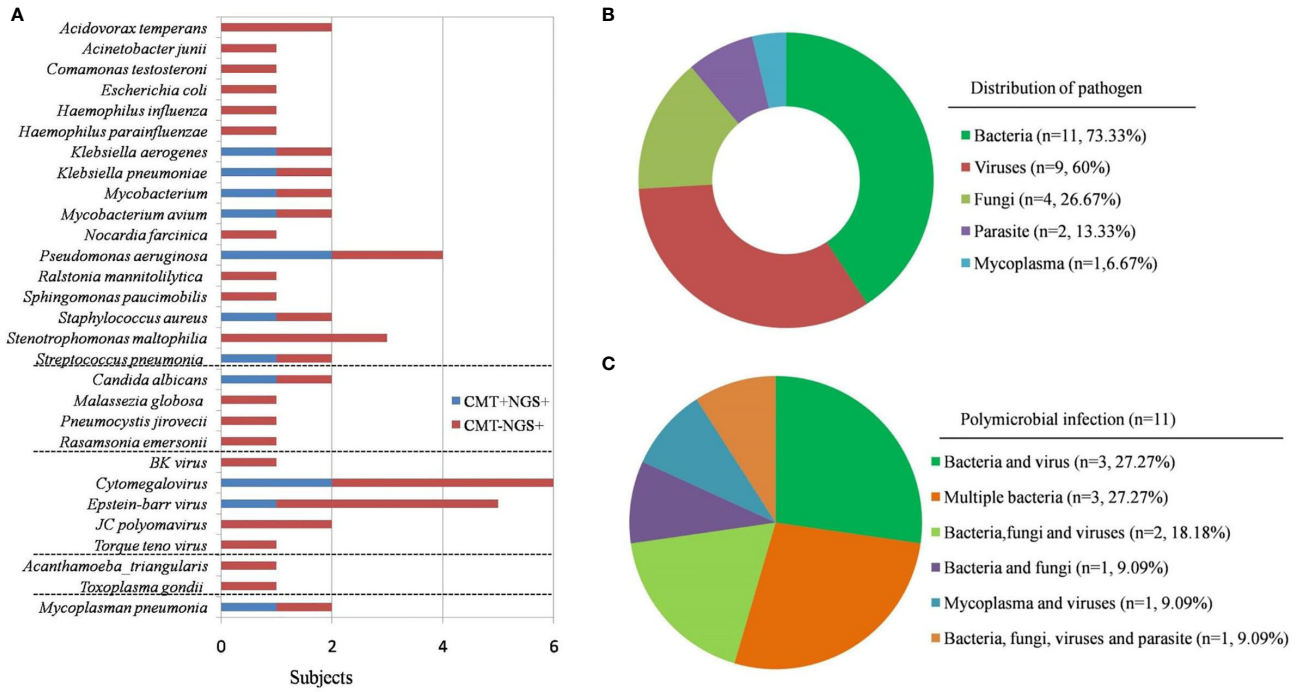

FIGURE 3 | Pathogens identified in PID patients using CMT and NGS. **(A)** The figure shows the number of subjects in whom each microbe was detected. Blue bars indicate microbes detected by CMT and predicted to be pathogens by NGS (CMT+NGS+). Red bars indicate microbes detected by NGS only (CMT–NGS+). **(B)** Distribution of pathogens in patients with PID. **(C)** Polymicrobial infection accounted for 73.33% of cases; different kinds of polymicrobial infection are shown.

P16 improved after treatment with isoniazid, rifampicin, pyrazinamide, ethambutol, and linezolid. P1 and P2 died within 1 year of *JC virus* infection. Pathological analysis of the liver lesions in patient P6 were diagnosed as EBV-associated leiomyosarcoma; the patient is still alive and awaiting HSCT. P13 died from severe infection caused by multi-drug resistant *Pseudomonas aeruginosa*. Some patients (P1-P6, P10-P13, P16) routinely received oral sulfamethoxazole to prevent *pneumocystis carinii pneumonia* or bacterial infection. In P11 and P12, itraconazole was taken orally to prevent fungal infection.

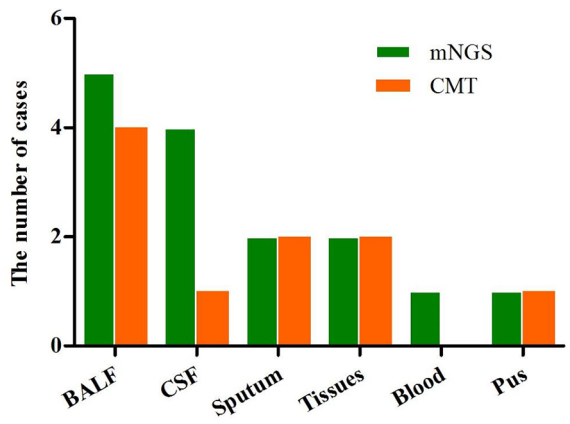

FIGURE 4 | Overall sensitivity of mNGS and CMT in the different sample types.

DISCUSSION

There are several causes why it is difficult to identify pathogens in PID. First, some patients have received antibiotics empirically before pathogen identification, thereby reducing the chances of a positive culture. Second, multiple concurrent infections and atypical pathogens are common in patients with PID; therefore, it can be difficult to identify the target pathogen using traditional cultures, PCR, immunofluorescence analysis, serological tests, and other CMT. Furthermore, because a considerable number of patients with PID are deficient in antibody production, antibody-dependent detection methods are ineffective. Also, infection-related clinical and imaging manifestations may appear atypical in patients with PID. These characteristics pose a severe challenge to pediatricians, or even PID experts, with respect to establishing specific etiology and choosing appropriate tests. Therefore, rapid and effective laboratory tests are necessary.

Reports on the use of mNGS to identify infections in patients with PID are scarce, although many previous studies recommend the use of mNGS for pathogen detection in patients with secondary immunodeficiency disease and in intensive care patients with life-threatening infections (5, 12–14). In November

2020, Chinese experts published an expert consensus on the clinical application of China's mNGS technology for detecting infectious pathogens. They pointed out that for new, rare, and treatment-refractory infectious diseases, and for patients with immunocompromising disease, mNGS can improve the pathogen detection rate significantly and can be used as the first-line method of detection (19).

Several studies report low positivity rates by conventional methods such as culture (20, 21). This study highlight the capacity of mNGS to detect pathogens that are unidentifiable by CMT. For example, P1–P3 had a suspected CNS infection, although no pathogens were detected by CMT. However, mNGS identified *JC virus* and *toxoplasmosis*. Moreover, most patients with no pathogenic evidence of infection had received empirical antibiotics. This led to side effects such as intestinal dysbacteriosis, liver and kidney damage. The high incidence of *Stenotrophomonas maltophilia* (3/15) in this study maybe evidence for carbapenem abuse, in addition to immunodeficiency itself. Therefore, mNGS may be used to exclude fever as a sign of infection. Implementation of mNGS as a rule-out strategy may reduce the abuse of antibiotics, as well as the duration of antibiotic therapy, in these patients.

Previous studies demonstrate the utility of mNGS for detecting pathogens that cause (or may cause) encephalitis, bloodstream infections, lower respiratory tract infections, and focal infections, in different sample types (5–14). Multiple case reports describe the use of mNGS to identify viruses, bacteria, fungi, and parasites in CSF and brain tissue (8, 10). A previous study shows that compared with CMT, mNGS has a sensitivity of 73%, a specificity of 99%, a positive predictive value of 81%, and a negative predictive value of 99%, for pathogen detection in CSF (22). Another multicenter study found that mNGS of CSF samples represents a potential step forward in the diagnosis of meningoencephalitis, thereby guiding earlier and more targeted treatments for neuroinvasive infections, and identifying emerging infections and disease phenotypes (9). In terms of the effectiveness and sensitivity of mNGS in different samples, we found that the sensitivity of mNGS for detecting pathogens in CSF, BALF, and blood samples was higher than that of CMT. Also, a recent study showed that mNGS is more sensitive than CMT for detecting pathogens in BALF, tissue, blood, and sputum samples (23).

Several studies report that polymicrobial infections are one of the most important features of immunocompromised hosts (24–26). Several studies highlight the potential of mNGS to supplement routine diagnostic methods in cases of co-infection with multiple pathogens (5, 12, 13). Consistent with previously studies, we found that 11 patients had polymicrobial infections; in our hands, mNGS showed clear advantages over other methods with respect to high detection efficiency and speed (results obtained in 48 h).

Patients with PID are susceptible to infection by opportunistic pathogens or rare pathogens (1, 2). We found that mNGS was superior to CMT for identification of opportunistic pathogenic microorganisms such as *JC polyomavirus*, *Pneumocystis jirovecii*, and *Stenotrophomonas maltophilia*, and for detection of causative agents that either had a relatively low culture rate or took a long time to culture (e.g., *Mycobacterium*). *Stenotrophomonas maltophilia* is a common nosocomial opportunistic pathogen in immunocompromised patients and in patients with Job's syndrome (27, 28). In the present study, P12 developed a liver abscess caused by co-infection by *Stenotrophomonas maltophilia* and *Staphylococcus aureus*; and was relieved by treatment with linezolid and compound Sulfamethoxazole. Toxoplasmic encephalitis (TE) is one of the most important neurological opportunistic infections in T cell-deficient patients. *Toxoplasma gondii* is difficult to culture and microscopic examination of CSF is insensitive. TE can be identified by cranial imaging, PCR, and antibody detection methods, whereas it highly depends on the doctor's experience. In addition, the serum anti-toxoplasma antibody is always absent in PID patients (8). In patient P3, we diagnosed TE by mNGS; this patient recovered after targeted sulfamethoxazole treatment. The number of standard unique reads for *Mycobacterium* and *Pneumocystis jirovecii* was lower than that for other pathogens. It is difficult to obtain circulating genomic DNA from intracellular bacteria (29); thus, low reads can also provide clues for diagnosis. A previous study demonstrates that for patients suspected of having TB, the diagnostic ability of mNGS was similar to that of Xpert; and mNGS may be more suitable for scarce samples such as CSF (30).

However, mNGS still has some limitations. The host genetic background and the background caused by bacterial contamination creates "noise", there are no uniform standards for detailed experimental procedures, it is difficult to distinguish infection from colonization, there is a lack of standardization with bioinformatics analysis processes regarding cut-off values, and data interpretation is tricky (22). It is extremely challenging to confirm the true pathogenicity of multiple pathogen infections, or opportunistic pathogen infections, detected by mNGS in patients with PID. In fact, the CMT also facing this problem. The microbe results must be interpreted in the context of the clinical situation, including immune defect itself, clinical manifestations and therapeutic effects.

This study has certain limitations. First, the sample size was relatively small, and we did not categorize specific types of PID as such a rare disease. Further studies should recruit a larger cohort and categorize patients with specific types of PID to better explore and describe the pathogen spectrum and its relationship to deficiency subtype. Second, some of the patients in our study received antibiotics therapy, which will affect the diagnostic performance of both mNGS and CMT. Additionally, RNA was not sequenced in all specimens for economic factors and CMT include common RNA virus testing; therefore, rare causes of infection (such as RNA viruses) may have been partially missed. Finally, this was a single center retrospective study so there is potential for bias. Besides, it is often unclear whether microbes detected using mGNS are contaminant, colonizer or pathogen, which need more study especially in PID.

In conclusion, our data suggest that mNGS results showed higher agreement with the clinical diagnosis and took shorter time. mNGS is a strong candidate for pathogenic diagnosis in PID patients with suspected severe, mixed, complicated, or treatment-refractory infections. The mNGS technology has marked diagnostic potential for complementing routine

diagnostic methods, particularly in the context of opportunistic pathogens and mixed infections, or in cases with negative CMT results. However, the identification of pollutants, colonizers or pathogens still needs more study, which is also a problem faced by CMT.

DATA AVAILABILITY STATEMENT

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2017) in National Genomics Data Center (Nucleic Acids Res 2021), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number CRA004615 that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

WT planned the study, analyzed the data, and wrote the paper. YA conceived and designed the study, supervised the study, and revised the paper. YZ, CL, LZ, and ZZ collected samples and carried out clinical diagnosis, treatment and follow up of patients. XT and XZ revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.696403/full#supplementary-material

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Characterization of a Cohort of Patients With LIG4 Deficiency Reveals the Founder Effect of p.R278L, Unique to the Chinese Population

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DNA ligase IV (LIG4) deficiency is an extremely rare autosomal recessive primary immunodeficiency disease caused by mutations in LIG4. Patients suffer from a broad spectrum of clinical problems, including microcephaly, growth retardation, developmental delay, dysmorphic facial features, combined immunodeficiency, and a predisposition to autoimmune diseases and malignancy. In this study, the clinical, molecular, and immunological characteristics of 15 Chinese patients with LIG4 deficiency are summarized in detail. p.R278L (c.833G>T) is a unique mutation site present in the majority of Chinese cases. We conducted pedigree and haplotype analyses to examine the founder effect of this mutation site in China. This suggests that implementation of protocols for genetic diagnosis and for genetic counseling of affected pedigrees is essential. Also, the search might help determine the migration pathways of populations with Asian ancestry.

Keywords: LIG4 deficiency, primary immunodeficiency disease, founder effect, haplotypes, mutation

1 INTRODUCTION

DNA double-strand breaks (DSBs) are a deleterious form of DNA damage that can result in loss or rearrangement of genomic material, both of which lead to cell death or carcinogenesis (1–3). DSBs are induced by ionizing radiation, but they also arise as intermediates during normal endogenous processes such as DNA replication, and meiotic and V(D)J recombination. In mammalian cells,

non-homologous DNA end joining (NHEJ) is the major mechanism for repairing DSBs (4). NHEJ involves at least six proteins: Ku70, Ku80, DNA-PKcs, DNA ligase IV, XRCC4, and Artemis. DNA ligase IV (LIG4) associates with XRCC4 during the final rejoining step of NHEJ (5).

LIG4 deficiency (OMIM 606593) is an extremely rare autosomal recessive disorder caused by mutations in the LIG4 gene. It is characterized by microcephaly, growth retardation, developmental delay, dysmorphic facial features, variable immunodeficiency, pancytopenia, a predisposition to malignancy, and pronounced clinical and cellular radiosensitivity (6).

The LIG4 gene maps to chromosome 13q33-q34; it contains two exons and comprises four domains: the DNA-binding domain (DBD), the nucleotidyltransferase domain (NTD), the oligo-binding domain (OBD), and the XRCC4-binding domain (XBD) (7). Our previous report identified p.R278L (c.833G>T) as a unique mutation site present in the majority of Chinese cases. We predicted that the p.R278L mutation is a hot spot or a founder effect in a Chinese population (8).

The founder effect is a genetic variation that occurs when a new population is created from a small number of individuals in a larger population (9). Ideally, the frequency of alleles in a population is distributed randomly between progeny; however, selection, variation, or inbreeding can affect the frequency of alleles in the progeny. A series of genetic markers that are inherited together through generations is called a haplotype; a haplotype demonstrates high linkage, resulting in little or no separation during meiotic recombination (10). Usually, the most common haplotype represents the polymorphism of most individuals within a population, which tends to be inherited as a whole by the offspring. Haplotype analysis can help confirm whether there is a founder effect in a mutation (11).

Here, we describe the clinical, immunological, and genetic characteristics of patients with LIG4 deficiency and investigate the phenotypic and mutation spectrum of the LIG4 deficiency to determine whether the p.R278L mutation is descended from a common ancestor *via* the founder effect.

2 MATERIALS AND METHODS

2.1 Patients

Initially, all patients enrolled in the study were suspected of having combined immunodeficiency based on clinical manifestations, examination findings, and clinical laboratory results. Eight patients with LIG4 deficiency (P8 to P15) were recruited from the Children's Hospital of Chongqing Medical University between 2016 and 2020. Seven patients (P1 to P7) that harbored the p.R278L mutation in the previous study were included (8); therefore, 15 patients were enrolled. The relevant clinical data are summarized in **Table 1A**. The assessment criteria for child growth and development published by WHO were used as a reference (12). Permission to participate in the study was provided by the patients' families (all of whom provided informed consent), and the study was approved by the Medical Ethics Committee of Children's Hospital of Chongqing Medical University.

2.2 Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly drawn heparin-treated blood by Ficoll density gradient centrifugation, as described previously (8).

2.3 Immunological Function Analyses

2.3.1 Lymphocyte Subsets

Whole blood was used for standard flow cytometry multicolor analysis; staining of lymphocyte surface markers was performed after red cell lysis, as described previously (13). A total of 20 subpopulations were examined to analyze T and B lymphocyte subsets.

2.3.2 T Cell Receptor Excision Circles and Kappa-Deleting Recombination Excision Circles

During T cell receptor rearrangement, excised DNA fragments create TRECs. During B cell maturation, KRECs are generated during kappa-deleting recombination allelic exclusion and isotypic exclusion of the light chain. TRECs reside within the chromosome, whereas KRECs are excised from genomic DNA. Quantification of TRECs and KRECs was performed using DNA samples extracted from peripheral blood. Quantification of TRECs and KRECs was performed by nested and quantitative real-time reverse transcription polymerase chain reaction (PCR) (qRT-PCR) (14, 15).

2.3.3 CDR3 Spectratyping

Each T cell receptor (TCR) Vβ fragment was amplified using one of 23 Vβ-specific primers and a 5'FAM-labeled Cβ primer (16). The PCR products were sequenced by Sangon Biotech Company (Shanghai, China). The data were analyzed using Gene Mapper V3.5, and a scoring system was used to evaluate TCR Vβ diversity: a score <4 indicated a skewed subfamily (17, 18).

2.3.4 Assessment of Maternofetal T Cell Engraftment

To detect the presence of maternofetal T cell transfusion, DNA samples obtained from each patient and their mother were subjected to short tandem repeat (STR) analysis by Kindstar Global Gene Technology Company (Wuhan, China).

2.3.5 Proliferation of T Cell and B Cell

PBMCs were incubated with 1.25 μl/ml CFSE (Invitrogen) at 37°C. After 10 min, the cells were washed twice at 4°C with 5 ml Roswell Park Memorial Institute (RPMI) medium containing 10% fetal bovine serum (FBS). Cells were then resuspended in 600 μl of RPMI/10% FBS and seeded into 96-well plates along with 5 μg/ml phytohemagglutinin (PHA), 10 μg/ml lectin from pokeweed mitogen (PWM), and the same volume of RPMI for 72 h. After staining with CD3-PerCP (clone: HIT3a, BioLegend), CD4-PE-Cy7 (clone: RPA-T4, BioLegend), CD8-PE (clone: RPA-T8, BioLegend), and CD19-APC (clone: HIB19, BioLegend) antibodies, cells were analyzed and examined by flow cytometry (8).

2.4 LIG4 Mutation Analysis

Genomic DNA was extracted from peripheral blood leukocytes using a Gentra Puregene blood kit (Qiagen, Hilden, Germany). In some patients (P1–P4, P7, P10–P15), NGS of the family was

TABLE 1A | Anthropometric data and clinical characteristics.

	Sex	Age of onset (months)	Age of diagnosis (months)	Family history	Gest/ weeks	BW/ s.d. (kg)	Anthropometric Data				Clinical Features								
							Age of examination (months)	OFC/s.d. (cm)	Height/s.d. (cm)	Weight/ s.d. (kg)	Form of onset	Development delay	Facial dysmorphia	Immunodeficiency	Etiology of infections	Malignancy	Others	Outcome
P1	F	11	18	_	38	−3.5(2.2)	18	−4.0 (40)	−3.0 (72)	−3.0(7.0)	Pneumonia, diarrhea	+	_	Chronic diarrhea, respiratory infection, thrush, sepsis, otitis media, onychomycosis	*Streptococcus pneumoniae*, CMV	Non-Hodgkin lymphoma	Pancytopenia	Die of malignancy At 3 years
P2	F	5	35	_	40	−1.5(2.9)	35	−4.0 (42)	−4.0 (80)	−4.5(7.5)	Pneumonia, diarrhea	+	_	Chronic diarrhea, respiratory infection, thrush, otitis media	*Streptococcus pneumoniae*, *Candida albicans*	_	Neutropenia and anemia, Colonoscopy: colitis	Die of severe Pneumonia at 3.5 years
P3	F	12	18	_	38	−3.0(2.4)	30	−5.0 (40)	−5.0 (72)	−5.0(7.0)	Pneumonia, diarrhea	+	_	Chronic diarrhea, pneumonia, otitis media, peritpnitis	*Streptococcus pneumoniae*, *Haemophilus influenza*	_	Anemia and thrombocytopenia, Inguinal hernia	Die of severe pneumonia at 3 years
P4	M	2	3	_	38	−2.5(2.5)	3	−3.0 (37)	−3.0 (55)	−3.5(3.0)	Pneumonia, diarrhea	+	_	Chronic diarrhea, severe pneumonia, ARDS	N/A	_	Anemia, atrial septal defect	Die of severe pneumonia at 4 months
P5	F	8	24	_	38	−4.0(2.0)	30	−4.5 (41)	−3.0 (80)	−5.5(6.0)	Diarrhea	+	+	Chronic diarrhea, severe Pneumonia	*Salmonella typhimurium*, *Pneumocystis carinii*	_	Pancytopenia, Phenylalanine dysmetabolism	Die of severe Pneumonia at 2.4 years
P6	F	10	18	+	39	−3.0(2.4)	21	−4.5 (40)	−5.0 (67)	−4.0(6.5)	Diarrhea	+	+	Chronic diarrhea, recurrent pneumonia	*Salmonella typhimurium*, *Enterobacter aerogenes*	_	AIHA and thrombocytopenia	Die of severe pneumonia at 2.5 years
P7	M	6	23	_	40	−2.5(2.6)	23	−7.0 (40)	−5.0 (70)	−5.5(5.0)	Pneumonia, Anemia	+	_	Chronic diarrhea, severe pneumonia, BCG infection, thrush	*Salmonella typhimurium*, CMV, acid-producing *Klebsiella*	–	Pancytopenia, Cytomegalovirus retinitis, and blindness	Die of severe pneumonia at 2 years
P8	M	8	18	_	38	−1.5(2.9)	18	−4.0 (42)	−4.0 (72)	−3.0(8.0)	Pneumonia, diarrhea	+	_	Chronic diarrhea, Recurrent pneumonia	*Salmonella typhimurium*, EBV	_	Pancytopenia	Die of severe pneumonia and diarrhea at 3 years
P9	F	2	12	+	36	−3.5(1.9)	13	−3.5 (40)	−3.0 (67)	−2.0(7.5)	Lymphocytopenia, thrombocytopenia	+	_	Chronic diarrhea, thrush and purpura	*Salmonella typhimurium*, *Candida albicans*	_	Pancytopenia	Die of HLH and sepsis at 3 years after HSCT
P10	M	1	12	_	38	−1.0(3.0)	11	−5.5 (39)	−2.0 (70)	−2.0(7.5)	BCG infection	_	_	Chronic diarrhea, respiratory infection and BCG infection	*Salmonella typhimurium*, BCG	_	Pancytopenia	HSCT at 2 years Survive
P11	M	4	28	_	38	−3.0(2.4)	35	−4.5 (43)	−3.0 (85)	−3.0(10.0)	Diarrhea	_	_	Chronic diarrhea	N/A	_	Pancytopenia	Survive
P12	M	23	36	_	38	−3.0(2.4)	36	−5.5 (42)	−3.0(84.5)	−4.0(8.5)	Neutropenia	_	+	Chronic diarrhea, severe pneumonia, wart	*Salmonella typhimurium*, EBV, CMV, *Candida albicans*	Large B-cell lymphoma	Neutropenia and anemia	Survive
P13	F	7	20	_	38	−2.0(2.5)	17	−4.0 (40)	−3.0 (72)	−5.0(5.0)	Diarrhea, thrush	+	+	Chronic diarrhea, severe pneumonia, thrush	*Enterobacter aerogenes*, *Salmonella typhimurium*, EBV, CMV, Parainfluenza virus	_	Pancytopenia	Survive
P14	M	22	48	_	40	−1.5(3.0)	48	−2.0 (47)	−1.0 (100)	1.0(18.5)	Pancytopenia	_	_	_	_	_	Pancytopenia	Survive
P15	M	6	12	+	38	-2.5(2.6)	12	−7.5 (36)	−3.0 (67)	−4.5(5.0)	Diarrhea	_	_	Chronic diarrhea, severe pneumonia, BCG infection	*Moraxella catalae*, BCG	_	AIHA, subglottic stenosis	Survive

Anthropometric data stated as Zscores (standard deviation from population mean for age and sex), actual measurements in brackets. F, female; M, male; Gest, gestation; BW, birth weight; OFC, occipitofrontal circumference; s.d., standard deviation; (–), negative; (+), positive; AIHA, autoimmune hemolytic anemia; ARDS, Acute Respiratory Distress Syndrome; BCG, Bacille Calmette-Guerin; CMV, cytomegalovirus; HSCT, hematopoietic stem cell transplantation; HLH, Hemophagocytic lymphohistiocytosis; N/A, not available.

performed firstly. The filtration of the WES data includes DNA library preparation, enrichment and sequencing of targeted genes, and bioinformatics analysis (MyGenostics, Beijing, China), as previously described (8). Genes associated with primary immunodeficiency diseases and other immune-related diseases had been updated according to the IUIS PID Classification Committee. In this study, four steps were used to select the potential pathogenic mutations in downstream analysis (i): Mutation reads should be more than 5, and mutation ration should be no less than 30% (ii); The mutations should be removed, when the frequency of mutation was more than 5% in 1,000 g, ESP6500, and Inhouse database (iii); The mutations should be dropped, if they were in InNormal database (MyGenostics) (iV); The synonymous mutations should be removed, when they were not in the HGMD database. After that, the rest mutations should be the potential pathogenic mutations for further analysis and judgment based on clinical phenotypes and phenotypic databases (OMIM and ClinVar). All mutations identified by NextSeq 500 sequencing were confirmed by Sanger sequencing. The coding exons and exon-intron boundaries of LIG4 were amplified by PCR. Both strands of the amplified PCR products were sequenced by Sangon Biotech Company. Whole-exon sequencing of P13 suggested that there might be copy number variation of exon. Therefore, the normal control samples, proband, and family samples were conducted by fluorescence quantitative PCR, and the copy number of the second exon of the target gene LIG4 was detected with the ALB gene as the internal reference gene. The pathogenicity of mutations was evaluated by four algorithms [PROVEAN (http://provean.jcvi.org/index.php), SIFT (http://sift.jcvi.org/), MutationTaster (http://www.mutationtaster.org/), and CADD (https://cadd.gs.washington.edu/snv)]. A scaled CADD PHRED of greater or equal 20 indicates the 1% most deleterious mutations in human genome. The frequency of mutations was searched in ChinaMap (http://www.mbiobank.com), gnomAD (https://gnomad.broadinstitute.org/), and DDBJ (https://www.ddbj.nig.ac.jp/index-e.html). The potential structural impact of the novel mutations was predicted by PymoL 2.1 program (https://pymol.org/2/).

2.5 The Expression of the Mutant Protein

Full-length wild-type (WT) LIG4 cDNAs was ordered from Youbio Biotech Company (Changsha, China). Mutant LIG4 cDNAs (p.R278L and p.R278H) were constructed by PCR mutagenesis and then subcloned into the p3xFlag-CMV-7.1 vector. In the overexpress system, HEK293T cells were transfected with 0.5 μg plasmids (WT, p.R278L, p.R278H), respectively. Cells were harvested 24 h after transfection, and then the expression of LIG4 was tested by Western blot with anti-LIG4 (EPR16531, Abcam) or anti-Flag antibody (2B3C4, proteintech).

2.6 Haplotype Analysis for LIG4 p.R278L Mutation

Haplotype analysis was performed to determine whether the LIG4 p.R278L mutation represents a founder mutation. Single-nucleotide polymorphisms (SNPs) were analyzed by Guoke Biotechnology Company (Beijing, China) using an Illumina Infinium® Human Omni 2.5-8 v1.3 Bead-Chip (Illumina, San Diego, CA, USA). Eight SNPs [rs9301287, rs9559278, rs1931348, rs1931349, rs2391626, rs915047, KGP10207473 (rs9514825), and KGP9393242 (rs9520821)] within a genomic region of 500 kb around the LIG4 p.R278L mutation (rs104894421) and with linkage disequilibrium (LD) values >0.2 were selected based on the results of Plink linkage analysis. Variations between Chinese Han South (CHS) and Chinese Han Beijing (CHB) populations were downloaded from the 1000 Genomes Project (https://www.internationalgenome.org/) and used as "normal sample haplotype" information. The frequencies of the identified haplotypes were analyzed using the Haploview 4.2 program (https://www.broadinstitute.org/haploview/). A binomial probability formula was used to calculate the probability of a mutation occurring recurrently as a *de novo* event in the same haplotype.

2.7 Estimating the Age of the Mutation

To better understand its history, the age of the LIG4 p.R278L mutation was estimated using the DMLE 2.3 program (http://www.dmle.org/). This software uses the Markov chain Monte Carlo algorithm for Bayesian inference of mutation age based on the observed LD at multiple genetic markers. The population growth rate was set as 0.025, with an intergenerational time interval of 25 years. The disease sample ratio was 0.002, which is the software default parameter.

3 RESULTS

3.1 Clinical Characteristics of Chinese Patients With LIG4 Deficiency

All 15 patients were from different families. Eight were male and seven were female. There was no evidence of potential skewing in the sex ratio. The clinical characteristics are listed in **Table 1A**. P6, P9, and P15 had a family history of early death or failed pregnancy (the older sister of P6 died of pneumonia at the age of 1 year, the older sister of P9 died of recurrent fever and diarrhea at the age of 8 months, and the mother of P15 had a previous pregnancy with embryo growth arrest). All were full-term infants, although five were small for their gestational age (SGA; i.e., birth weight 2 standard deviations below average).

The average age of symptom onset was 8 months (range, 1–23 months), and the median time of diagnosis was 18 months. The most common onset manifestations (soon after birth or later in life) included diarrhea, pneumonia, thrush, and BCG infection; two cases (P9 and P14) presented initially with hemolytic anemia.

Every patient except P14 suffered from chronic diarrhea, which aggravated their nutritional status and potentially contributed to growth failure. Pneumonia was the second most common and deadly type of infection. Salmonella was cultured from the stools of seven patients with diarrhea. The main etiological agent of respiratory tract infections was bacteria, including *Streptococcus pneumoniae*, *Enterobacter aerogenes, Moraxella catarrhiae, Acid-Producing Klebsiella*, and *Haemophilus influenzae*. The copy number of cytomegalovirus (CMV) in the blood of four patients

was high (P1, P7, P12, and P13). BCG infection was suspected in P10 and P15; the BCG vaccination site was ulcerated, and adjacent lymph nodes were enlarged.

Growth failure was evident in all patients. Occipitofrontal circumference, weight, and height were significantly below normal values (p <0.05). Data on head circumference at birth were scarce; however, our estimates were low because almost every patient presented at our hospital with a head circumference >3 SD below the population mean. A previous study in mice shows that LIG4 is essential for neuronal cell development. Consequently, most patients presented with short stature and microcephaly. Ten cases (P1–P9 and P13) showed developmental delay; all failed to achieve milestones of child development for their age group. P12 and P13 had a large nose, with a prominent nasal bridge as the facial dysmorphism (**Figure 1**).

All patients exhibited cytopenia (**Table 1B**). Leukocytes were the most affected cell type (11/15), followed by red blood cell. Three patients presented with pancytopenia. Bone marrow aspiration revealed active bone marrow hyperplasia with no morphological abnormalities, while showed failure bone marrow in biopsy. Notably, P14 manifested with cytopenia; initially, the bone marrow results led us to suspect that this patient had "myelodysplastic syndrome" (MDS) or "aplastic anemia" (AA). There was some evidence of autoimmunity. Coomb's test was positive in four patients during the early course of the disease (P5, P6, P7, and P9). Two patients were positive for thyroid peroxidase antibodies (P9 and P10).

P12 presented with hemocytopenia, characterized by chronic diarrhea and pneumonia. Abdominal imaging and biopsy (of the sigmoid colon) at the age of 4 years confirmed EBV-positive diffuse large B cell lymphoma (non-germinal center origin). Chemotherapy was not a treatment option due to pulmonary fungal infection and poor nutritional status.

Prior to diagnosis of LIG4 deficiency, P7 and P9 received steroids and rituximab to treat autoimmune hemolytic anemia (AIHA) and thrombocytopenia. P14 received cyclosporine for hemocytopenia; this patient was refractory to immunosuppressive therapy. Due to repeated infections, most patients received a variety of antibacterial or antiviral drugs, and some received antifungal or antituberculous drugs. However, P1–P8 died or stopped treatment without transplantation due to marked exacerbation of pulmonary infections and respiratory failure. Hematopoietic stem cell transplantation, the only effective radical cure for AIHA, was performed for P9 and P10. The donors were their respective parents (haploidentical donors) due to HLA matching difficulties; both engrafted successfully. After transplantation, mycophenolate mofetil, tacrolimus, MTX, and steroids were used for GVHD prophylaxis. Unfortunately, P9 developed recurrent fever 3 months after transplantation, thought to be due to EBV-driven post-transplantation lymphoproliferative disease and hemophagocytic lymphohistiocytosis (HLH). She received the HLH-2008 chemotherapy regimen (including two doses of VP16) but died of sepsis 3 months later. Notably, HCT did not cure microcephaly and neurodevelopmental delay in P10. The remaining patients (P11–P15) have not received HCT, but continue to survive; all receive IVIG and oral co-trimoxazole to prevent infection (standard treatments after a diagnosis of LIG4 deficiency).

3.2 Immune Characteristics

Immunological function analyses were also performed (**Table 1B**). Flow cytometry analysis of peripheral blood showed a significant reduction in the absolute numbers of CD19+ B cell and CD3+ T cell; however, NK cell (T-B-NK+ phenotype) counts were near-normal. Seven of them (P8, P9, P10, P11, P12, P14, and P15) were analyzed in detail. Two patients (P8 and P15) exhibited a marked increase in the number of γδ T cell compared with healthy children. IgG levels in six of the 14 patients (except the P11) were significantly lower than the normal reference value (p <0.05). Others had normal levels of IgG; however, they were tested after receiving intravenous immunoglobulin. The TRECs and KRECs count was significantly below the detection limit, although the lymphocyte counts in P8 and P15 were not that low (19). STR analysis of eight patients ruled out maternofetal transfusion (P8–P15).

Analysis of TCR-Vβ diversity was performed in five newly enrolled patients (P8, P12, P13, P14, and P15). Most TCR-Vβ subfamilies exhibited monoclonal or oligoclonal peaks, and TCR

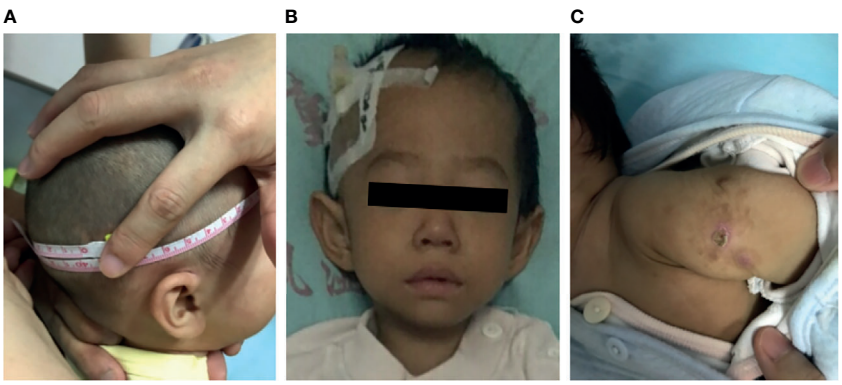

FIGURE 1 | Clinical data for patients with LIG4 deficiency. **(A)** Microcephaly in patient P9. **(B)** Facial dysmorphism in patient P13. **(C)** BCG scar ulceration and exudation in patient P10.

TABLE 1B | Hematological and immune investigations.

	Hematological test results					Lymphocyte subset cells/ul					Immunoglobulins (g/L)			TRECs (copies/reation)	KRECs (copies/reation)	Autoimmune antibodies
	WBC (10^9/L)	Neut	Lymph	Hb (g/L)	PLT (10^9/L)	CD3 +	CD3+ CD4+	CD3+ CD8+	CD19 +	CD16+ CD56+	γδT	IgG	IgA	IgM			
P1	2.55	68%	25%	90	30	164	14	88	2	69	N/A	1.92	0.01	1.45	N/A	N/A	N/A
P2	3.09	78%	16%	73	173	138	52	60	1	272	N/A	13.8 (IVIG)	0.067	0.22	N/A	N/A	N/A
P3	3.88	83%	11%	92	45	255	31	189	0	50	N/A	0.087	0.049	0.389	N/A	N/A	N/A
P4	5.05	35%	58%	78	283	1,020	216	464	31	1,916	N/A	0.921	<0.067	0.123	32	18	N/A
P5	2.89	56%	38%	97	7	513	22	394	0	16	N/A	9.17 (IVIG)	<0.067	0.663	10	7	Coomb's test (+)
P6	3.59	68%	30%	84	67	161	37	88	1	540	N/A	10.2 (IVIG)	<0.067	0.217	106	2	Coomb's test (+)
P7	3.25	27%	65%	95	80	1,739	316	1,265	285	1,165	N/A	<0.333	<0.067	<0.0417	N/A	N/A	Coomb's test (+)
P8	6.21	58%	35%	71	5	1,694	320	1,155	8	1,450	962	1.12	<0.0667	1.54	2	5	N/A
P9	2.2	80%	6%	103	3	78	18	23	19	178	62	6.7 (IVIG)	0.37	1.03	0	0	Coomb's test (+), TPOAb (+)
P10	2.15	74%	18%	95	276	170	23	63	1	94	112	8.24 (IVIG)	0.38	1.1	0	0	TPOAb (+)
P11	3.98	72%	20%	93	82	550	102	320	1	258	225	N/A	N/A	N/A	3	0	N/A
P12	1.69	69%	10%	90	200	151	72	43	12	190	55	6.6 (IVIG)	<0.001	<0.001	14	0	N/A
P13	1.42	14%	69%	70	40	1,012	177	556	1	495	N/A	1.46	0.51	1.54	0	0	N/A
P14	2.01	39%	29%	105	75	450	110	210	10	312	105	9.71	0.67	0.72	0	0	N/A
P15	7.4	51%	41%	105	370	1,288	230	510	3	1,737	1,081	5.4 (IVIG)	1.07	0.485	0	0	N/A

WBC, white blood count; Neut, neutrophil count; Lymph, Lymphocyte count; Hb, hemoglobin; PLT, platelet count; TRECs, T cell receptor excision circles; KRECs, kappa-deleting recombination excision; TPOAb, thyroid peroxidase antibodies; N/A, not available. Red means above the reference range, and blue means below the reference range.

repertoire complexity was limited, a finding similar to that in our previous report (8). P14 and P15 exhibited less skewed TCR diversity than healthy controls, suggestive of less severe impairment of V(D)J recombination and TCR function. In those age-matched healthy controls, the majority of the 23 TCR-Vβ subfamilies exhibited a Gaussian curve with 6–9 peaks, reflecting a polyclonal Vβ repertoire. The frequency of skewed TCR-Vβ subfamilies in the patients was higher than that in the healthy control (**Figure 2**).

When PBMCs from patients were stimulated with PHA for 3 days, the percentages of proliferating CD4+ and CD8+ T lymphocytes were 0.2 and 1.9% in P8; 2.78 and 4.33% in P11; 3.79 and 1.67% in P12; and 1.1 and 8.3% in P14, respectively. CFSE fluorescence histograms generated by flow cytometry revealed no obvious peak with respect to cell division. The percentages of proliferating CD4+ and CD8+ T lymphocytes in the PBMCs population from the normal control were 71.9 and 65.6%, respectively, with obvious peaks in cell division. Taken together, these data suggest that T cell proliferation in the LIG4 deficiency patients was severely impaired. B cell proliferation was analyzed only in P11. Proliferation of CD19+ B cell after stimulation by PWM was 4.93% in the patient and 47.8% in the normal control.

3.3 Genetic Characteristics

Patients with LIG4 deficiency came from 11 different provinces in China. Mutations in the LIG4 gene were detected by either Sanger or next-generation sequencing. According to the clinical manifestations of microcephaly, immune deficiency, and autosomal recessive inheritance pattern, LIG4 was finally identified as the sole pathogenic gene. No other PID genes in the 2019 update of the IUIS IEI classification were found in each patient. Fourteen different mutations were identified, three of which have not been described previously. Most were compound heterozygous mutations, while P3 and P8 harbored a homozygous p.R278L. Most mutations were predicted to be "Deleterious," "Damaging," "Disease causing," or "Prediction disease causing" by different algorithms. Frameshift mutations and copy number variants cannot evaluated by CADD. All CADD PHRED score of the missense or non-sense mutations were >20 and regarded as deleterious except p.T9I. The allele frequency of p.T9I was about 20% in ChinaMap and was predicated as SNP by Mutation Taster. Mutation p.R278L was found in ChinaMap (6/21176) and gnomAD (1/1558, East Asian) but not in DDBJ or other population (**Table 2**).

Since recurrent infection is the predominant feature in these patients, and antibody deficiency and lymphocytopenia are present as a combined immunodeficient immunophenotype, we hypothesized that these mutations are loss of function. Since it was difficult to get enough primary cell from patients, we tried to build the KO fibroblast cell line several months ago. However, the cell line did not proliferate after LIG4 gene knockout, and we failed to get the KO clone. We transfected the mutant LIG4 (p.R278L and p.R278H) to HEK293 as an overexpression system and found that the mutant protein expressed comparably to wild type. To visualize the function of R278 at the amino acid level, we constructed a diagram to describe the structure of the DBD domain of LIG4, in which

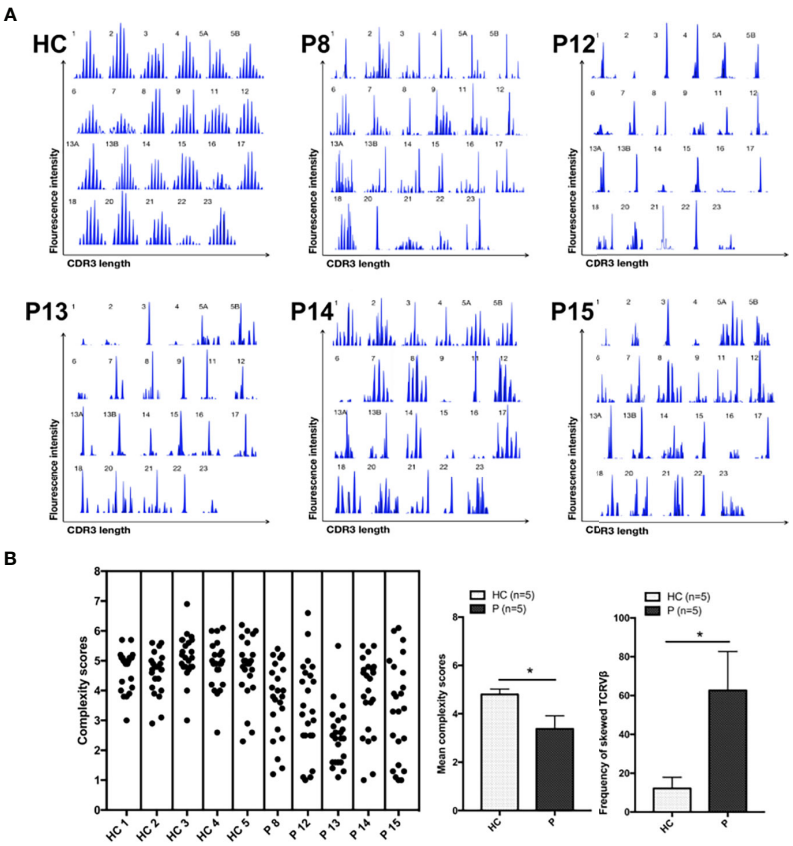

FIGURE 2 | TCR-Vβ analysis. **(A)** All 23 TCR-Vβ subfamilies in the healthy controls exhibited a Gaussian distribution. CDR3 size distribution of the TCR-Vβ subfamilies in patients P8, P12, P13, P14, and P15. **(B)** The complexity scores for each healthy control subject (left) and patient (right) are shown. The mean (range) complexity scores of the patient and control groups are shown in the histogram. The frequencies of TCR-Vβ subfamilies with skewed CDR3 length patterns are shown in the histogram, as determined by a complexity score <4. HC, healthy control subjects; P, patients. *$p < 0.05$.

the main role of R278 is to bind ATP (**Figure 3**). The map reveals that mutations in R278 affect the activity of enzymes by altering the spatial conformation and the ability to bind ATP. LIG4 deficiency patients carrying the p.R278L mutation were concentrated in the Yangtze River valley of China. All mutation sites are shown in the simulation diagram (**Figure 4**) (20–37). Mutation p.R278L is a hot spot found only in Chinese patients. It is worth mentioning that the p.R278L mutation was also detected in P13; however, the ratio of the copy number of exon 2 of the LIG4 gene to that of the normal control was about 0.5 (**Supplementary Figure**), suggesting large deletion of heterozygosity in exon 2 of the LIG4 gene on the allelic chromosome. Another common genotype is p.K424RfsX20, which occurs in non-Chinese cases.

3.4 Shared Haplotype and Estimated Age of the Mutation

A total of eight unrelated LIG4 deficiency patients harboring the p.R278L mutation were included for haplotype analysis (P1, P4, P5, P6, P8, P9, P11, and P12). Haplotypes were characterized using a set of eight SNPs flanking the c.833G>T (p.R278L) mutation, with a length of approximately 2.3 kb (**Supplementary Table** and **Figure 5**). Using the Haploview program, a block of LD was located beside the LIG4 gene; three haplotypes were identified in the patients and five in the healthy control population. Haplotype analysis shows that haplotype GGACTACT was the most common in the patient group (53.8%), but occurred in only 19.5% of the CHS and 17.5% of the CHB population (**Figure 6**). The haplotype of the p.R278L mutation site is significantly different from that of the normal allele.

DMLE+ 2.3 was used to analyze the age of the mutation; DMLE+ 2.3 provides the posterior distribution probability of the age of the mutated haplotype (38). Based on an intergeneration time of 25 years, it predicted that the age of the mutation is 353 generations (95% credible set; 217–454 generations), i.e., 8,825 years (95% credible set: 5,425–11,350 years) (**Figure 7**).

4 DISCUSSION

According to the IEI classification standard updated by IUIS in 2019, LIG4 deficiency is a type of severe combined immunodeficiency (SCID) defined by CD3/CD19 lymphopenia, which affects cellular

TABLE 2 | Genetic characteristics.

	Nucleotide change	Protein change	Father	Mother	Province of Origin	Prediction				Frequency (ChinaMap)	
						PROVEAN	SIFT	Mutation Taster	CADD PHRED	Allele Frequency	Count
P1	c.833G>T	p.R278L	p.R278L		Zhejiang	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.1271-1275delAAAGA	p.K424RfsX20		p.K424RfsX20		N/A	N/A	Prediction disease causing	N/A	0.000472233	10/21176
P2	c.833G>T	p.R278L		p.R278L	Sichuan	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.1271-1275delAAAGA	p.K424RfsX20	p.K424RfsX20			N/A	N/A	Prediction disease causing	N/A	0.000472233	10/21176
P3	c.833G>T	p.R278L	p.R278L		Guizhou	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.833G>T	p.R278L		p.R278L		Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
P4	c.833G>T	p.R278L	N/A	N/A	Shanxi	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.2113G>T	p.E705X				N/A	N/A	Disease causing	34.0	N/A	N/A
P5	c.833G>T	p.R278L	p.R278L		Tianjin	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.26C>T	p.T9I		p.T9I		Deleterious	Damaging	Ploymorphism	18.9	0.2066696	4377/21176
	c.1142-1143delCT	p.L382EfsX4		p.L382EfsX4		N/A	N/A	Prediction disease causing	N/A	0.000236116	5/21176
P6	c.833G>T	p.R278L	p.R278L		Beijing	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.935delC	p.P313HfsX19		p.P313HfsX19		N/A	N/A	Prediction disease causing	N/A	N/A	N/A
P7	c.833G>T	p.R278L	p.R278L		Inner Mongolia	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.2134-2135delTA	p.I712AfsX5		p.I712AfsX5		N/A	N/A	Prediction disease causing	N/A	N/A	N/A
P8	c.833G>T	p.R278L	p.R278L		Sichuan	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.833G>T	p.R278L		p.R278L		Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
P9	c.833G>T	p.R278L		p.R278L	Jiangxi	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.1271-1275delAAAGA	p.K424RfsX20	p.K424RfsX20			N/A	N/A	Prediction disease causing	N/A	0.000472233	10/21176
P10	c.1296A>T	p.K432N	p.K432N		Henan	Deleterious	Damaging	Disease causing	23.7	N/A	N/A
	c.1672C>T	p.Q558X		p.Q558X		N/A	N/A	Prediction disease causing	40.0	N/A	N/A
P11	c.833G>T	p.R278L		p.R278L	Henan	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.34T>A	p.S12T	p.S12T			Neutral	Damaging	Disease causing	21.1	0.00056679	12/21176
	c.2710C>T	p.Q904X	p.Q904X			N/A	N/A	Disease causing	38.0	N/A	N/A
P12	c.833G>T	p.R278L		p.R278L	Hubei	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.1271-1275delAAAGA	p.K424RfsX20	p.K424RfsX20			N/A	N/A	Prediction disease causing	N/A	0.000472233	10/21176
P13	c.833G>T	p.R278L		p.R278L	Guizhou	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	loss exon2	loss exon2				N/A	N/A	N/A	N/A	N/A	N/A
P14	c.980T>G	p.I327S		p.I327S	Henan	Deleterious	Tolerated	Disease causing	26.7	N/A	N/A
	c.2585_2586del	p.H862RfsX6	p.H862RfsX6			N/A	N/A	Prediction disease causing	N/A	N/A	N/A
P15	c.833G>T	p.R278L	p.R278L		Hunan	Deleterious	Damaging	Disease causing	26.0	0.00028334	6/21176
	c.1271-1275delAAAGA	p.K424RfsX20		p.K424RfsX20		N/A	N/A	Prediction disease causing	N/A	0.000472233	10/21176

N/A, not available.

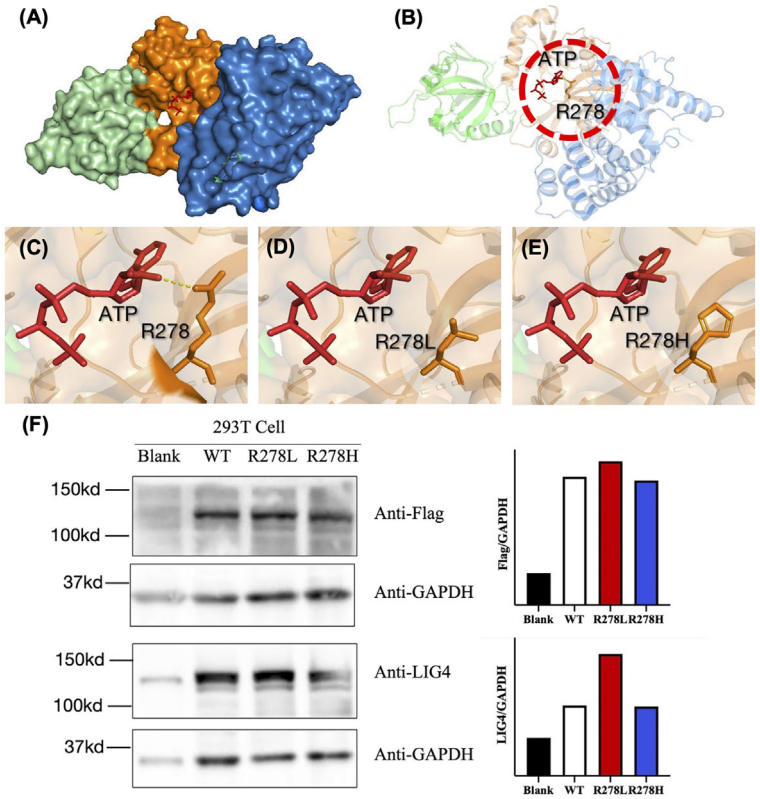

FIGURE 3 | **(A)** Structural characterization of the catalytic domain of human DNA LigIV (PDB ID code 3W5O). The domains are shown in blue (DBD), orange (NTD), and cyan (OBD). The structure surrounding by Arg-278 (indicated by the dashed circle in **(B)** is enlarged. **(C–E)** The interaction between ATP and amino acids R278, R278L, and R278H was drawn using a tool packaged in PymoL. **(F)** Western blot analysis of the LIG4 protein expression in the HEK293T cells transfected with WT or mutant LIG4 plasmid (p.R278L or p.R278H).

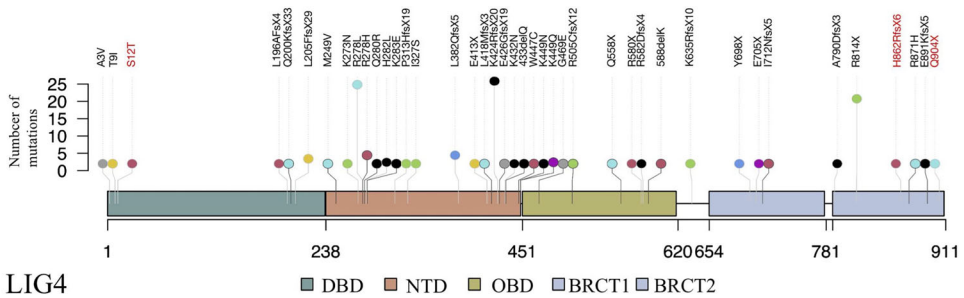

FIGURE 4 | Schematic presentation of the LIG4 gene structure and a summary of mutation sites. The novel mutation sites identified in this study are shown in red. The height of the line represents the number of mutations. DBD, DNA-binding domain; NTD, nucleotidyltransferase domain; OBD, oligo-binding domain; XBD, XRCC4-binding domain.

and humoral immunity (39). As such, LIG4 deficiency has a broad-spectrum phenotype that includes immunodeficiency, microcephaly, growth failure, facial dysmorphism, malignancy predisposition, and cellular sensitivity to ionizing radiation. Additional features include bony deformations such as syndactyly and congenital hip dysplasia (6).

Due to combined immunodeficiency, approximately three quarters of LIG4 deficiency patients suffer from recurrent

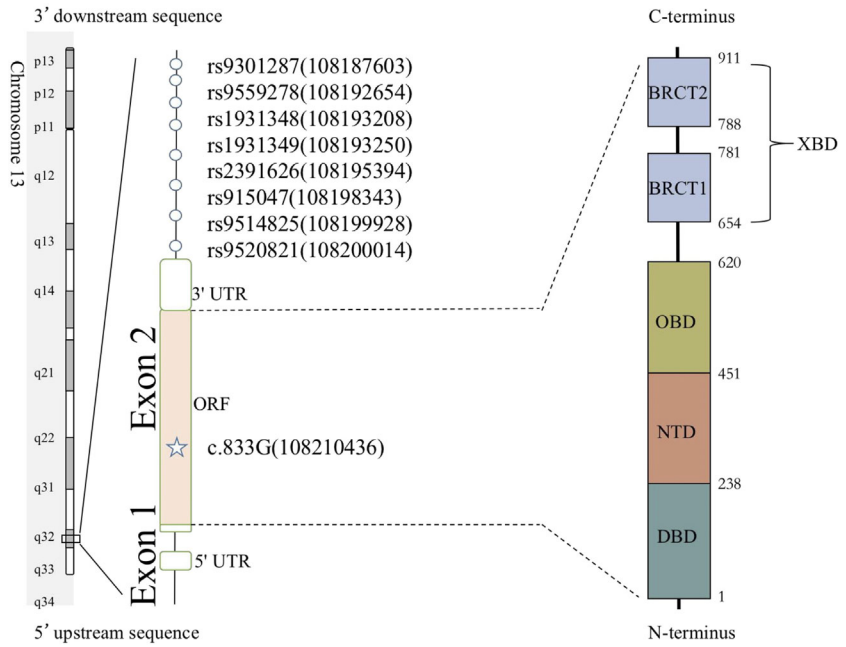

FIGURE 5 | Eight SNPs were used to reconstruct the haplotypes for the LIG4 mutation c.833G. The positions of genetic markers (shown in brackets) were defined according to GRCh38.

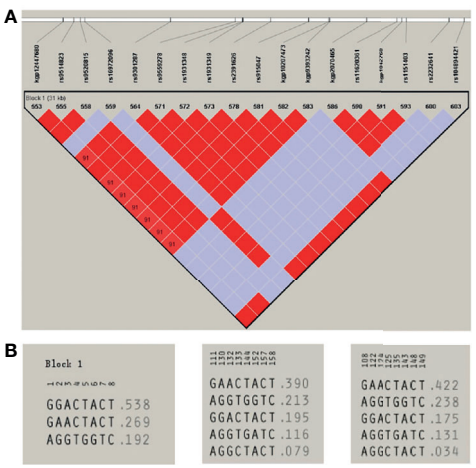

FIGURE 6 | **(A)** Haploview-generated linkage disequilibrium (LD) patterns for the LIG4 mutation c.833G>T (p.R278L) and the predicted block structure. The rate of LD is represented by different colors (the highest rate of LD is red, the lowest in purple). **(B)** Potential haplotypes of the eight selected SNPs genotyped in our study in Patients (left) and in CHS (middle) and CHB (right) populations.

infections, with varying degrees of severity. Among them, sinopulmonary infections are the most common (20). By contrast, the most common manifestation in our patient cohort was severe gastrointestinal tract infection. Chronic diarrhea and recurrent pneumonia were common at onset in almost all patients, leading to multiple hospital admissions and failure to thrive. Causative pathogens included bacteria, viruses, and fungi. Intestinal infection by *Salmonella typhimurium* is a unique manifestation in Chinese patients, indicating that gastrointestinal prophylaxis is of great importance. BCG is a unique pathogen associated with live attenuated vaccinations. Thus, a delayed BCG vaccination, or an IPV vaccination plan within 1 month after birth, should be considered as a replacement for the live attenuated vaccination plan.

It is classified as a severe immunodeficiency, and our patients mainly present severe immunodeficiency for loss of LIG4 function, whereas P14 had no history of recurrent or severe infection; the only manifestation was cytopenia. Initially, we suspected "MDS" or "AA" and so treated the patient with cyclosporine for 2 years, even though two compound heterozygous mutation sites in LIG4 gene had been detected before he came to our hospital. The p.I327S mutation in P14 was also detected in another LIG4 deficiency patient with pancytopenia and lymphoma, whereas the other mutation (p.H862RfsX6) has not been reported previously. After hospitalization at our center, laboratory tests confirmed the pathogenicity of these mutations by indicating a significant decrease in TRECs/KRECs and T lymphocyte proliferation, the TCR diversity is not so severely impaired as the other patients. The p.H862RfsX6 mutation located in the XBD causes a premature stop codon six amino acids downstream of the C-terminus of the LIG4 protein. Previous studies show a genotype-phenotype correlation with respect to the position of truncating mutations corresponding to disease severity. Transcripts encoding truncated proteins are predicted to be expressed at normal levels (21). We speculate that P14 present

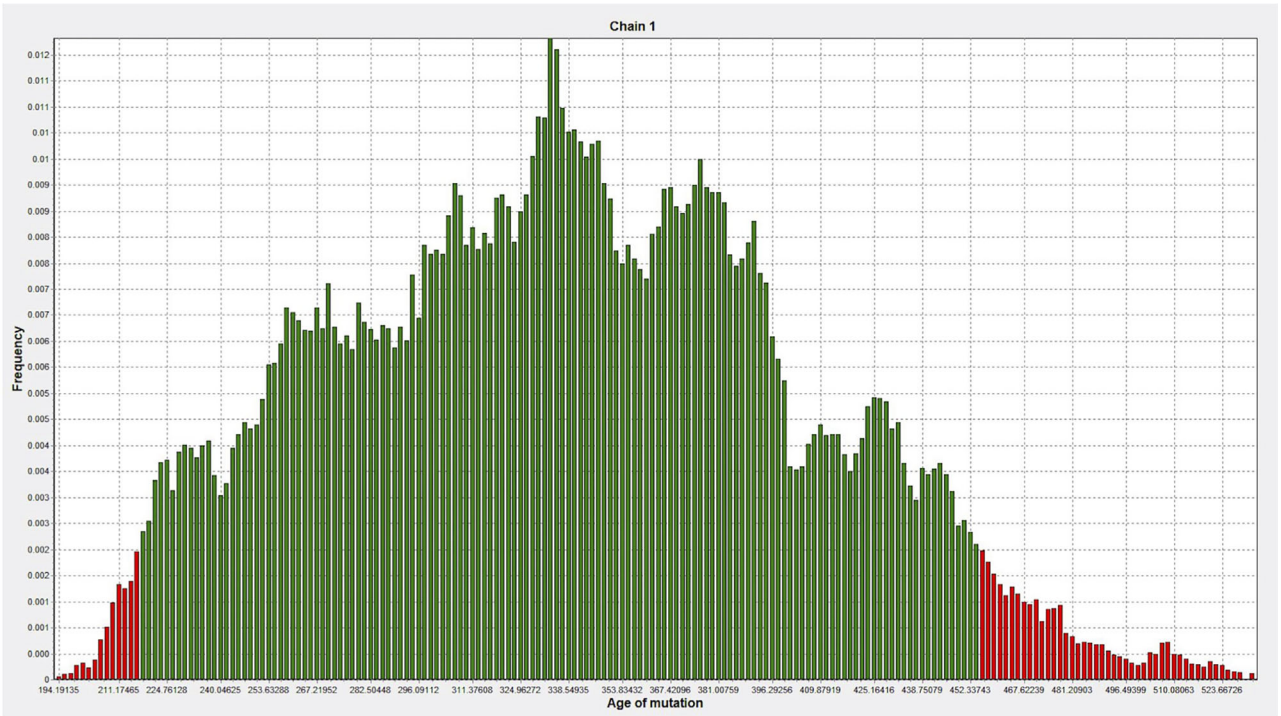

FIGURE 7 | The age of the mutation was simulated 100,000 times by DMLE+2.3. The abscissa represents the age of mutation, in units of generation (about 25 years), and the ordinate represents the frequency (Green, within the 95% confidence interval of posterior distribution; Red, outside the 95% confidence interval).

as hypomorphic phenotype since the mild clinical manifestation, less skewed TCR diversity, higher lymphocyte and immunoglobulin due to heterogenous p.I327S missense mutation.

Extreme growth failure and microcephaly is a common and early presentation of LIG4 deficiency patients. Murray et al. even screened some LIG4 deficiency patients for microcephalic primordial dwarfism before making a diagnosis of CID or SCID (21). This may be due to accumulation of DNA-DSB over time resulting in reduced lymphocyte production. Most of our patients were full-term infants, although they were SGA, which suggests intrauterine growth retardation. A previous study in mice shows that LIG4 is essential for neuronal cell development (40). LIG4 deficiency may lead to impaired prenatal differentiation of neuronal cell and result in microcephaly and developmental delay. Thus, significant microcephaly and growth restriction are regarded as the most prominent features of LIG4 deficiency patients. Developmental delay also occurred in five of our patients. "Bird-like" or "Seckel syndrome-like" traits are always observed in LIG4 deficiency patients (20). In this study, P12 and P13 had a large nose with a prominent nasal bridge; however, there were no other facial dysmorphisms such as a low anterior hairline, bilateral epicanthic folds, or up slanting palpebral fissures (6).

The symptomatic treatment of LIG4 deficiency patients includes long-term antibiotics, antiviral and antifungal chemoprophylaxis, immunoglobulin infusion, transfusion support, and avoidance of unnecessary exposure to ionizing radiation. HCT is a curative treatment for CID and SCID immunophenotypes and might reduce the risk of developing lymphoid malignancy. Of note, reduced-intensity conditioning regimens with low-dose Cyclosporin A should be considered due to radiosensitivity (6). However, HCT does not cure microcephaly or neurodevelopmental delay. Short stature and mild to moderate intellectual disability may remain. Extra social care is required to maintain a good quality of life, including attendance at a special school for intellectual disability (21). In our study, nine of the 15 patients died. In another cohort of LIG4 deficiency cases in China, four of seven died and two were lost to follow-up (37). There were another four patients in the other papers who were also lost to follow-up, and the possibility of death was high (35, 36). Thus in China, improvement of the early diagnosis and adequate treatment are necessary to improve the poor prognosis of LIG4 deficiency.

Due to disruption of V(D)J recombination, rearrangement of T and B lymphocyte receptors is aberrant, resulting in combined immunodeficiency and a skewed TCR-Vβ repertoire, as confirmed in the new cohort patients (P8–P15). Therefore, most LIG4 deficiency patients present with T-B-NK+ phenotype SCID, along with reduced antibody production. Flow cytometry analysis showed that reduced B cell counts are more pronounced than reduced T cell counts due to B cell development is more reliant on V(D)J recombination, maternofetal transfusion, or a spontaneous somatic reversion (8). However, STR confirmed no maternal engraftment in our eight patients (P8–P15), so maternofetal transfusion is not as common as in X-SCID patients. Also, there was no reduction in γδT cell. Previous studies argue that limited V

(D)J recombination activity may provide γδ T cell with a developmental advantage (22, 41). LIG4 has also been shown to be involved in immunoglobulin class switch recombination (6). Thirteen of 14 (93%) and nine of 14 (64%) patients had low IgG and IgA levels, respectively, whereas eight of 14 patients (57%) had normal IgM levels.

Genetic analysis revealed that most Chinese LIG4 deficiency cases harbor the p.R278L mutation, which is unique and represents a mutational hot spot in China. There is clinical evidence for the pathogenicity of this mutant site. Another mutation in the same codon, which causes a different amino acid substitution (p.R278H) (40, 42), was reported only in a non-Chinese ethnic population. According to the pathogenicity prediction software, both of these mutations cause functional loss by affecting binding to ATP, although this needs to be confirmed. The P3 and P8 with homogeneous R278L manifested as severe infection, very low T/B cell counts, low TRECs, as well as normal protein expression (**Figure 3F**), indication p.R278L as loss of function mutation.

The high frequency and the geographic clustering of the LIG4 p.R278L mutation may be a hot spot or even a founder mutation. According to the principle of LD, haplotypes reflect the genetic information at the mutation site since they tend to be passed on to offspring as a whole. For example, haplotype analysis revealed three founder mutations (BRCA1 c.68_69delAG, c.5266dupC, and BRCA2 c.5946delT) in the Ashkenazi Jewish population, and a recurrent F8 mutation (c.6046C>T) causing hemophilia A in a northern Italian population (43, 44). In this study, we constructed five different haplotypes to explore the origin of the alleles carrying the p.R278L mutation in our LIG4 deficiency patients. Haplotype analysis identified only three haplotypes in LIG4 deficiency patients, the most common being haplotype GGACTACT (53.8%); this haplotype was much less common in the controls (CHS, 19.5%; CHB, 17.5%). The different frequencies of haplotypes, along with reduced genetic diversity, are typical features of isolated and stable populations. Thus, it is plausible that the LIG4 p.R278L mutation frequency increased in China due to a local founder effect.

There has been much debate about the origin of mankind. In recent years, a large amount of genomic data has been obtained and accumulated. The completion of the human genome project in 2003, the launch of the 1000 Genomes Project in 2008, and the study of ancient DNA have provided new clues that explain the evolution of the genetic structure of populations (45, 46). The recent African origin model hypothesis states that *Homo erectus*, the common ancestor of early *Homo sapiens* or Archaic humans, originated in Africa and then spread from the continent to other parts of the world about 2 million years ago. India is one of the many crossroads in the history of mankind (47, 48). Interestingly, when we analyzed this haplotype (GGACTACT) among other ethnic groups from the 1000 Genomes Project, we found that Bangladeshi and Pakistani (PJL), as well as the Indian populations in the United States (GIH) and England (ITU), also had the highest frequency of this haplotype. Therefore, we cannot exclude that this mutation might have spread from South Asia *via* migration (49–51). However, no LIG4 deficiency patients of Bangladeshi, Pakistani, or Indian origin have been reported.

Regarding the age of the common ancestor, the DMLE estimates that the LIG4 p.R278L mutation is approximately 8,825 years old. This corresponds to the Neolithic age, the period during which agriculture began in settled communities. The distribution patterns of such founder mutations might help determine the migration pathways of populations with Asian ancestry (48, 49). However, estimating the age of founder mutations will always be an inexact endeavor. The true recombination and mutation history of the relevant chromosomal segments is unknown. Thus, further studies on the prevalence of shared mutations in Asian countries, and haplotype analyses in other ethnic groups, would be helpful if we are to trace the common ancestor.

This study has some limitations. We did not confirm the DNA-repair defect in primary cell due to lack of PBMCs from patients. We also did not confirm it by other strategy since the fibroblast cell line did not proliferate after LIG4 gene knockout as well as the technical difficulties of recombinant LIG4 enzyme activity analysis. It could be overcome by primary cell from a newly diagnosed patient in the future.

5 CONCLUSION

In summary, LIG4 deficiency is a rare disease with a broad spectrum of presentations. The severity fluctuates greatly. In China, improvement of the early diagnosis and adequate treatment are necessary to improve the poor prognosis of LIG4 deficiency, and HCT is urgent. Pedigree analysis and haplotype construction revealed conservation of a single haplotype surrounding the p.R278L mutation, suggesting that this allele has a common ancestor. The finding of a founder effect in a highly recurrent mutation in a rare disease suggests that implementation of protocols for genetic diagnosis and for genetic counseling of affected pedigrees is essential. Also, the search for new targeted therapies such as base editing should be prioritized.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in Genebank with the following accession numbers:

BankIt2490138 ZJ-R278L MZ766072
BankIt2490148 QWY-R278L MZ766073
BankIt2490148 QWY-E705X MZ766074
BankIt2490153 GYT-R278L MZ766075
BankIt2490153 GYT-I712Afs MZ766076
BankIt2490153 XJ-R278L MZ766077
BankIt2490153 TKY-R278L MZ766078
BankIt2490153 TKY-K424Rfs MZ766079
BankIt2490153 DCZ-R278L MZ766080
BankIt2490153 DCZ-K424Rfs MZ766081
BankIt2490153 DYC-I327S MZ766082
BankIt2490153 DYC-H862Rfs MZ766083

BankIt2489891 TSY MZ825311
BankIt2490111 TSY-K424Rfs MZ825312
BankIt2490126 SXY-R278L MZ825313
BankIt2490126 SXY-K424Rfs MZ825314
BankIt2491465 YR-T9I MZ825315
BankIt2491472 YR-R278L MZ825316
BankIt2491472 YR-L382Efs MZ825317
BankIt2491472 WYH-R278L+P313HfsMZ825318
BankIt2491472 ZHR-K432N MZ825319
BankIt2491472 ZHR-Q558X MZ825320
BankIt2491472 DCY-R278L MZ825321
BankIt2491472 LC-R278L MZ825322
BankIt2491472 LC-K424Rfs MZ825323
BankIt2491472 ZKY-R278L MZ825324
BankIt2491472 ZKY-P904X MZ825325
BankIt2491472 ZKY-S12T MZ825326

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XL, YA, and XZ designed experiments and analyzed the data. XL wrote the first draft of the manuscript and performed the experiments. YD provided control specimens from normal healthy children. QL, JJ, WT, LZ, JY, and XT contributed to scientific discussion, data interpretation, and revision of the manuscript. XZ designed the research, supervised the study, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.695993/full#supplementary-material

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Revealing Chronic Granulomatous Disease in a Patient With Williams-Beuren Syndrome Using Whole Exome Sequencing

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Blended phenotypes exhibited by a patient may present a challenge to the establishment of diagnosis. In this study, we report a seven-year-old Murut girl with unusual features of Williams-Beuren syndrome (WBS), including recurrent infections and skin abscesses. Considering the possibility of a second genetic disorder, a mutation screening for genes associated with inborn errors of immunity (IEI) was conducted using whole exome sequencing (WES). Analysis of copy number variations (CNVs) from the exome data revealed a 1.53Mb heterozygous deletion on chromosome 7q11.23, corresponding to the known WBS. We also identified a biallelic loss of *NCF1*, which indicated autosomal recessive chronic granulomatous disease (CGD). Dihydrorhodamine (DHR) flow cytometric assay demonstrated abnormally low neutrophil oxidative burst activity. Coamplification of *NCF1* and its pseudogenes identified a GT-deletion (ΔGT) at the start of exon 2 in *NCF1* (NM_000265.7: c.75_76delGT: p.Tyr26Hisfs*26). Estimation of *NCF1*-to-*NCF1* pseudogenes ratio using ΔGT and 20-bp gene scans affirmed nil copies of *NCF1* in the patient. While the father had a normal ratio of 2:4, the mother had a ratio of 1:5, implicating the carrier of ΔGT-containing *NCF1*. Discovery of a 7q11.23 deletion involving one *NCF1* allele and a ΔGT in the second *NCF1* allele explained the coexistence of WBS and CGD in our patient. This study highlights the capability of WES to establish a molecular diagnosis for a case with blended phenotypes, enabling the provision of appropriate prophylactic treatment.

Keywords: whole exome sequencing (WES), chronic granulomatous disease (CGD), Williams-Beuren syndrome (WBS), copy number variation (CNV), blended phenotypes, dual molecular diagnosis

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INTRODUCTION

Williams-Beuren syndrome (WBS) (OMIM ID: 194050) is a contiguous gene deletion syndrome inherited in autosomal dominant pattern. Most cases occurred sporadically with an estimated prevalence of 1/7,500 to 1/20,000 live births (1, 2). Patients with WBS typically exhibited distinct facial appearance, cardiovascular abnormalities and developmental delay (3). Other features including idiopathic infantile hypercalcemia, gastrointestinal problems, musculoskeletal defects, hypothyroidism and hernias were noted in some patients (4). This genetic disorder is caused by a heterozygous deletion of 1.5-1.8Mb on chromosome 7q11.23 that encodes approximately 26 to 28 genes (5). Phenotypic variability between WBS patients may complicate the establishment of diagnosis based on clinical symptoms. Hence, a comprehensive genetic testing is necessary to confirm the diagnosis, particularly in cases with unusual manifestations.

Our study involved a seven-year-old patient with blended phenotypes. The patient manifested typical WBS features such as aortic stenosis, inguinal hernia, subclinical hypothyroidism and learning difficulty with developmental delay. Nevertheless, she also had recurrent infections, diarrhea and skin abscesses since the age of one year. As WBS patients are not commonly associated with immune disorders, we suspected the possibility of a primary immunodeficiency. Primary immunodeficiencies, recently termed as inborn errors of immunity (IEI) are characterized by increased susceptibility to infections, autoimmune diseases, autoinflammatory disorders, allergies and malignancies (6). These disorders are caused by monogenic mutations in at least 430 genes (6, 7). Diagnosing IEI based on the clinical and immunological abnormalities can be challenging owing to their phenotypic and genetic heterogeneity (8). Since the application of next-generation sequencing (NGS), the molecular diagnostics of IEI was revolutionized, facilitating the continuous discovery of IEI-associated genes (6, 9).

Previously, we utilized whole exome sequencing (WES) to diagnose patients suspected with IEI and obtained molecular diagnoses in 46.7% of the cohort (10). Among the three NGS approaches, whole genome sequencing (WGS) has the highest diagnostic capacity compared to targeted gene panel and WES. Still, WGS is minimally used in the clinical settings because of its high cost and large data output. Although a targeted gene panel generates more manageable data, it restricts the identification of novel disease-causing genes. In light of the affordable cost and computational requirements, WES is reasonable for both diagnostic and research purposes of IEI (11). The likelihood of encountering incidental findings and variants of uncertain significance increases as the NGS coverage expands. Thus, NGS data should be interpreted meticulously to ensure accurate reporting of deleterious mutations. Herein, we identified the genetic etiology responsible for the unexpected features of WBS in our patient using WES.

MATERIALS AND METHODS

Study Subject

A seven-year-old Murut girl with WBS caused by a heterozygous deletion (cytogenetic analysis not shown) was recruited by the Institute for Medical Research. The patient was presented with unexpected phenotypes of WBS, i.e., multiple severe infections and abscess formation, raising clinical suspicion of a cooccurring genetic disorder. Written informed consent was collected from the patient and her parents upon blood sample collection. The study received approval from the Medical Research and Ethics Committee, Ministry of Health Malaysia (KKM/NIHSEC/P16-837) and adhered to the Declaration of Helsinki.

Exome Sequencing and Bioinformatics Analysis

Genomic DNA was isolated from peripheral blood mononuclear cells using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Exome capture was performed using Agilent SureSelect Human All Exon V5 (Agilent, USA) with target size of 50Mb. Paired-end reads of 101 base pairs (bp) were generated from a HiSeq 4000 sequencer (Illumina, USA) at mean coverage of 100x. Burrows-Wheeler Aligner-maximal exact matches (BWA-MEM) was used to align the sequencing reads to the human reference genome GRCh38 (12). Mapped reads were subjected to soft clipping of adapter sequences, duplicate marking and read sorting using Picard tools. Genome Analysis Toolkit (GATK) was used for base quality score recalibration followed by variant calling. Variants including single nucleotide variants (SNVs) and short insertions or deletions (indels) were annotated using web-based ANNOVAR.

Variant Prioritization

Annotated variants were primarily filtered against the known IEI-associated genes documented by the International Union of Immunological Societies in 2019 and 2021 (6, 7). Exonic and splice site variants resulting in missense, nonsense, frameshift and nonframeshift mutations were retained. Rare variants with allele frequency of 0.0001 or less as reported by Genome Aggregation Database (gnomAD) were further analyzed. The functional impact of the variants was evaluated using *in silico* pathogenicity prediction tools, namely Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2) and MutationTaster (13–15). Variants with tolerated or benign impact predicted by more than one variant effect predictor were excluded from analysis. Mode of inheritance and genotype-phenotype correlation were determined through extensive literature search. The variants were interpreted according to the standards and guidelines of American College of Medical Genetics and Genomics (ACMG) (16).

Copy Number Variation (CNV) Analysis

ExomeDepth was used to detect CNVs from WES data *via* read depth approach (17). Read counts were generated from the BAM file containing aligned reads. An optimized reference set was built by selecting the most correlated samples with the study subject from the in-house exomes. Each exon was assigned with a likelihood value that designates one of the copy number variable states, namely deletion, normal and duplication. The likelihood values across multiple exons were merged using hidden Markov model. Bayes factor, which represents the likelihood ratio of CNV to normal copy number was calculated. In this regard,

CNV with a high Bayes factor is more likely to be true positive. CNVs shared by the patient and control were excluded from analysis. CNVs on autosomes were interpreted based on findings reported in Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (DECIPHER) and Database of Genomic Variants (DGV) (18, 19). The potential disease-causing CNV was plotted to show the ratio between the observed and expected read depth.

Dihydrorhodamine (DHR) Flow Cytometric Assay

Neutrophil oxidative burst activity was evaluated using Phagoburst™ kit (Orpegen Pharma, Germany) according to the manufacturer's instructions. The heparinized whole blood sample was incubated with a stimulant, protein kinase C activator phorbol 12-myristate 13-acetate (PMA) at 37°C. A negative control was prepared by substituting the stimulant with wash solution. After 10 minutes of incubation, nonfluorescent dihydrorhodamine 123 substrate solution was added to every sample. Oxidation of dihydrorhodamine 123 to fluorescent rhodamine 123 upon reaction with reactive oxygen species (ROS) was measured by a BD FACSCanto™ II flow cytometer (Becton Dickinson, USA) using FlowJo™ v10. Neutrophils were gated to analyze the percentage of cells producing ROS during unstimulated and PMA-stimulated conditions.

Genomic DNA Amplification and Sequencing

Polymerase chain reaction using published primers 2LB2 and 2RB2 was performed to screen for the GT-deletion (ΔGT) at the beginning of exon 2 in *NCF1* (20). The amplicons were sequenced bidirectionally using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on a 3730xl DNA Analyzer (Applied Biosystems, USA). FinchTV was used to visualize the sequence chromatograms.

ΔGT and 20-bp Gene Scans

Gene scans were performed to estimate the ratio of *NCF1* and its two pseudogenes (21). ΔGT gene scan targets the ΔGT in exon 2, while 20-bp gene scan targets both ΔGT in exon 2 and 20-bp copy in intron 2. The primers used for ΔGT gene scan were 6-FAM-labeled p47-ΔGT-fwd and p47-ΔGT-rev primers. Whereas for 20-bp gene scan, primers p47-ΔGT-fwd and HEX-labeled p47-20bp-rev were used. Separation of fluorescently labeled fragments was performed on a 3730xl DNA Analyzer (Applied Biosystems, USA). The ratio of *NCF1* and its pseudogenes was determined by dividing the peak heights displayed on PeakScanner.

RESULTS

Case Presentation

The patient was the fifth child born to nonconsanguineous parents. Both parents and her four elder siblings were well with no known medical illnesses. Cytogenetic analysis was prompted before enrolment considering the characteristic facial features, revealing the diagnosis of WBS. However, this diagnosis did not explain the recurrence of infections and abscesses experienced by the patient since early childhood. Hence, she was recruited for further genetic analysis. She initially presented with fever, abdominal distension, diarrhea and limb stiffening at the age of one year. Blood culture and sensitivity test detected *Escherichia coli*, while cerebrospinal fluid fungal culture grew non-encapsulated yeast. She was treated as *Escherichia coli* sepsis and meningitis with ampicillin, metronidazole, cefepime, vancomycin, meropenem and amphotericin B. She was also noted to have abscesses on her left distal forearm and right popliteal area which were treated with cloxacillin. At the age of three years, she had prolonged fever with diarrhea and vomiting caused by *Salmonella typhi*, requiring a two-week course of cefuroxime. Until the age of five years, she was admitted for infectious diarrhea and treated with meropenem. One month later, she presented with a left knee abscess and right forearm cellulitis. Blood culture grew *Streptococcus* sp. In between the admissions, she also had multiple episodes of bronchopneumonia, requiring oxygen support and intravenous antibiotics. Laboratory screening tests encompassing full blood count assessment, lymphocyte subset enumeration and quantitation of immunoglobulin and complement levels were performed (**Table 1**). The test results showed no abnormalities except elevated IgE.

Bioinformatics Interpretation of Exome Sequencing

Exome sequencing generated 67,606,668 paired-end reads with 99.5% of them properly paired and mapped to the human reference genome GRCh38. A total of 23,364 variants were detected in the exons (n=23,249) and splice sites (n=115) (**Figure 1**). These variants comprised 97.5% of SNVs and 2.5% of indels. After filtering against stringent criteria, the total variants were reduced to five potential disease-causing point mutations (**Table 2**). One SNV was identified in each of the five genes, namely *CFHR4*, *IL7R*, *CFTR*, *ERCC4* and *G6PD*. However, the inheritance pattern and associated features of these monogenic disorders were inconsistent with the patient's zygosity and clinical presentation respectively.

Biallelic Loss of *NCF1*

ExomeDepth identified a total of 195 CNV calls in the patient, of which 19 were shared with the control and two were localized on sex chromosomes. The CNVs retained for further analysis encompassed 94 deletions and 80 duplications. The proportion of predicted CNVs with a size larger than 1 kilobases (kb) (63.8%) was higher compared to those shorter than 1kb (36.2%). A high Bayes factor of 1,100 was computed for the heterozygous deletion on chromosome 7q11.23 ranging from 73,303,201 to 74,836,526, corresponding to the WBS region (**Figure 2A**). The 1.53Mb deletion involved genes from *NSUN5* to *GTF2IRD2*, including *NCF1*. The second allele of *NCF1* was found to be deleted, resulting in biallelic loss of *NCF1*.

TABLE 1 | Blood test results of the patient.

Parameter	(Unit)	Value	Age-matched reference range
Full blood count			
Red blood cells	($x10^{12}$/L)	5.0	4.0-6.0
Hemoglobin	(g/dL)	12.6	11.0-18.0
Hematocrit	(%)	43.1	35.0-60.0
MCV	(fL)	87.1	80.0-99.9
MCH	(pg)	25.5	27.0-31.0
MCHC	(g/dL)	29.3	33.0-37.0
RDW	(%)	16.5	11.6-13.7
White blood cells	($x10^9$/L)	14.3	4.5-10.5
Lymphocytes	($x10^9$/L)	6.7	1.2-3.4
Monocytes	($x10^9$/L)	2.6	0.1-0.6
Granulocytes	($x10^9$/L)	5.0	1.4-6.5
Platelets	($x10^9$/L)	784.0	150.0-450.0
Lymphocyte subsets			
$CD3^+$ T cells	($x10^6$/L)	3,300	1,400-2,000
$CD3^+/CD4^+$ Th cells	($x10^6$/L)	1,328	700-1,000
$CD3^+/CD8^+$ Tc cells	($x10^6$/L)	2,211	600-900
$CD19^+$ B cells	($x10^6$/L)	1,004	300-500
$CD3^-/CD16^+56^+$ NK cells	($x10^6$/L)	222	200-600
Immunoglobulins			
IgG	(g/L)	18.70	5.20-15.60
IgA	(g/L)	2.27	0.54-3.60
IgM	(g/L)	3.51	0.13-2.40
IgE	(kU/L)	139	<40
Complements			
C3	(g/L)	2.00	0.50-0.90
C4	(g/L)	0.47	0.10-0.40

MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; Th cells, helper T cells; Tc cells, cytotoxic T cells; NK cells, natural killer cells; Ig, immunoglobulin.

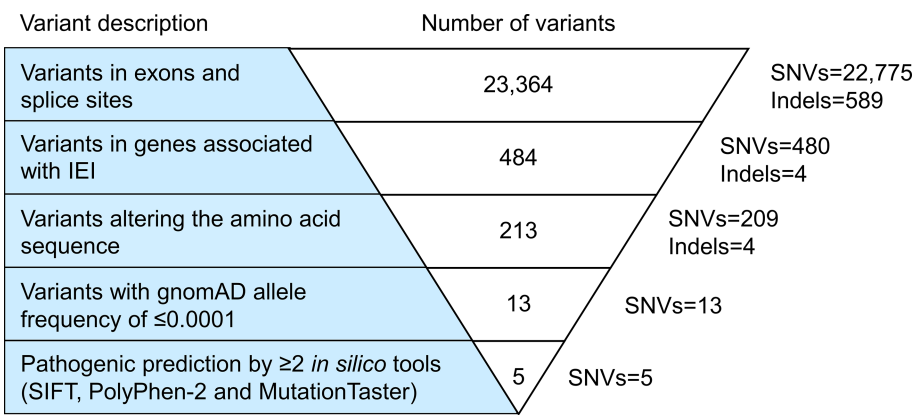

FIGURE 1 | Variant filtering strategy. All variants harbored in the exonic regions and splice sites were filtered based on the known IEI-associated genes, variant class, allele frequency and variant functional impact. Five potential causative single nucleotide variants (SNVs) fulfilled the filtering criteria. However, the inheritance pattern and associated features of these genetic disorders did not match the zygosity and clinical symptoms of the patient respectively. IEI, inborn errors of immunity; SNVs, single nucleotide variants; Indels, small insertions or deletions.

Impaired Neutrophil Oxidative Burst Activity

In response to PMA, the patient showed profoundly low intensity of rhodamine 123 as compared to a distinct shift in the control (**Figure 2B**). Decreased oxidation of DHR in the patient indicated defective oxidative burst activity of NADPH oxidase.

Validation of ΔGT in *NCF1*

Nonallele-specific amplification displayed overlapping traces of GTGT-containing *NCF1* and ΔGT-containing pseudogenes in the control and parents (**Figure 3A**). Conversely, only a sequence of *NCF1* pseudogenes was shown in the patient, indicating a ΔGT in *NCF1* (NM_000265.7: c.75_76delGT: p.Tyr26Hisfs*26). ΔGT gene scan detected fragments of two sizes, i.e., 196-bp fragment

TABLE 2 | List of nonsynonymous single nucleotide variants (SNVs) found in the inborn errors of immunity (IEI) genes after variant filtering.

Chr	Gene	RefSeq:exon:nucleotide change:amino acid change	Inheritance pattern (zygosity)	dbSNP	*In silico* prediction				Allele frequency				ACMG classification
					SIFT	PolyPhen-2	MutationTaster	CADD phred	1000 Genomes All	1000 Genomes East Asian	gnomAD All	gnomAD East Asian	
1	*CFHR4*	NM_006684.5: exon5: c.620C>T: p.S207F	AR/AD (Het)	rs1296597051	D	PD	N	18.5	0	0	0	0	Likely benign
5	*IL7R*	NM_002185.5: exon4: c.460C>T: p.H154Y	AR (Hom)	rs199727195	D	PD	D	22.5	7.0E-05	3.0E-03	8.5E-05	1.0E-03	Uncertain significance
7	*CFTR*	NM_000492.4: exon14: c.1865G>A: p.G622D	AR (Het)	rs121908759	D	D	D	27.0	3.8E-04	1.0E-03	1.2E-04	5.6E-04	Pathogenic
16	*ERCC4*	NM_005236.3: exon11: c.2545C>G: p.Q849E	AR (Het)	rs374186605	T	PD	D	21.9	7.7e-05	2.0E-03	8.8E-05	1.1E-03	Likely benign
X	*G6PD*	NM_000402.4: exon6: c.682C>T: p.R228C	XL (Het)	rs137852330	D	D	D	27.6	2.7E-04	0	3.3E-05	7.2E-05	Likely benign

Chr, chromosome; AR, autosomal recessive inheritance; AD, autosomal dominant inheritance; X-linked inheritance; Het, heterozygous; Hom, homozygous; D, deleterious; T, tolerated; PD, possibly damaging; N, polymorphism; SIFT, Sorting Intolerant From Tolerant; PolyPhen-2, Polymorphism Phenotyping v2; CADD, Combined Annotation Dependent Depletion; gnomAD, genome aggregation database; ACMG, American College of Medical Genetics and Genomics.

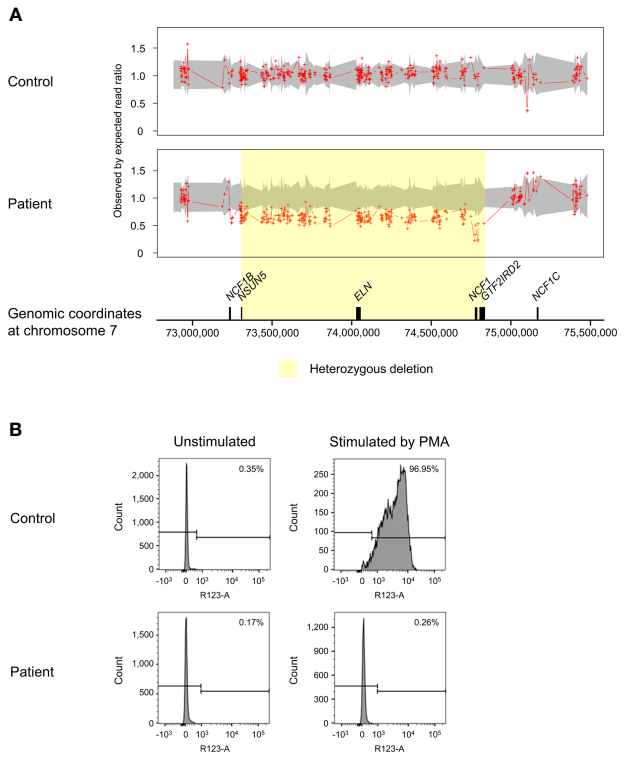

FIGURE 2 | Copy number variation (CNV) analysis on chromosome 7q11.23 and assessment of NADPH oxidase activity. **(A)** The patient was found to have a biallelic deletion of *NCF1* localized within the 1.53Mb heterozygous deletion associated with Williams-Beuren syndrome (WBS) (shaded in yellow). The breakpoints of WBS deletion occurred from chromosome 7:73,303,201-74,836,526, affecting 27 genes. Biallelic loss of *NCF1* leads to autosomal recessive chronic granulomatous disease (CGD). Aortic stenosis observed in the patient is consistent with the haploinsufficiency of *ELN*. Ratio of 1.5 indicates one-copy duplication, 1.0 indicates normal copy state, 0.5 indicates one-copy deletion and 0 indicates two-copy deletion. Grey shade represents the 95% confidence interval of expected read ratio. Each red plus symbol indicates the genomic position being probed. Size of genes (black-filled boxes) was approximate and not drawn to scale. **(B)** Assessment of neutrophil oxidative burst activity using dihydrorhodamine (DHR) flow cytometry assay. Upon stimulation by phorbol 12-myristate 13-acetate (PMA), the patient showed no change in the fluorescence intensity of rhodamine 123 (right panel). This result is consistent with CGD that lacks normal neutrophil oxidative burst activity. The percentage in the histograms indicates the proportion of neutrophils that produced reactive oxygen species (ROS). PMA, phorbol 12-myristate 13-acetate.

reflected *NCF1* pseudogenes with ΔGT and 198-bp fragment reflected *NCF1* with GTGT. The proportion of GTGT and ΔGT was found to be 2:4 in the control and father (**Figure 3B**). However, the patient had a single peak of *NCF1* pseudogenes, confirming the absence of wild-type *NCF1*. The mother had a ratio of 1:5, signifying the carrier of ΔGT-containing *NCF1*. In 20-bp gene scan, 411-bp fragment denoted the product of *NCF1* with GTGT and a single 20-bp stretch while 429-bp fragment denoted the product of pseudogenes with ΔGT and a duplication of 20-bp stretch. Ratios of *NCF1* and its pseudogenes obtained from ΔGT gene scan were consistent with that of 20-bp gene scan (**Figure 3C**).

DISCUSSION

In this study, we reported a case with dual molecular diagnosis of WBS and chronic granulomatous disease (CGD). Repeated infections and formation of skin abscesses in our patient raised the suspicion of an IEI. We performed initial screening for germline short variants associated with IEI using WES, but detected no causative variants matching the clinical presentation. Further analysis involving CNV detection from exome data uncovered a biallelic loss of *NCF1*, suggesting CGD. This finding was corroborated by DHR assay that showed remarkably low neutrophil oxidative burst activity. DHR assay is commonly used to diagnose CGD owing to its quantitative measurement of NADPH oxidase activity and high sensitivity (22). However, abnormal DHR oxidation in complete myeloperoxidase deficiency and Rac2 deficiency may lead to false-positive CGD diagnosis (23, 24). Thus, genetic analysis is useful to confirm the diagnosis of CGD.

Oxidative burst activity of NADPH oxidase, which involves ROS production is essential for the elimination of invading pathogens (25). Defects in one of the five subunits of NADPH oxidase will impair the release of ROS, resulting in CGD (26). Due to ineffective clearance of microbes, CGD patients are predisposed to recurrent infections and excessive inflammation (22). This IEI was estimated to affect 1/200,000 to 1/250,000 newborns, with majority of the cases caused by X-linked *CYBB* mutations (27). Monogenic defects in *CYBA*, *CYBC1*, *NCF1*, *NCF2* and *NCF4* led to the less common form of CGD inherited in autosomal recessive pattern (28, 29).

Mutation detection in *NCF1* can be tricky due to the presence of two pseudogenes that share at least 98% of DNA sequence similarity with the gene (30). Prominent differences, including GTGT at exon 2 and single 20-bp stretch at intron 2 distinguished *NCF1* from its pseudogenes that contain a ΔGT and duplicated 20-bp stretch respectively (31). Taking these two features into account, Dekker et al. had established a reliable gene scan method to determine the ratio of *NCF1* and its pseudogenes (21). The ΔGT and 20-bp gene scans revealed *NCF1* deficiency caused by a ΔGT in our patient as opposed to a normal ratio of 2:4 and a carrier ratio of 1:5 in the father and mother respectively. Deletion of GT in *NCF1* led to a frameshift followed by premature termination at amino acid residue 51, generating a truncated $p47^{phox}$. Unusually high incidence of ΔGT in unrelated patients was thought to arise from unequal crossing-over between *NCF1* and its highly homologous pseudogenes (31, 32). Clinical symptoms of WBS and CGD in the patient can be explained by a WBS deletion involving one *NCF1* allele and a ΔGT in the second *NCF1* allele. As the mother was found to be the carrier of ΔGT-containing *NCF1*, we hypothesized that the patient inherited the *de novo* 7q11.23 deletion including *NCF1* from her father (**Figure 4**). The genetic diagnosis has enabled our patient to be treated with continuous prophylactic antibiotics and antifungal to prevent severe infections.

Patients with defective NADPH oxidase are increasingly susceptible to infections caused by catalase-positive microorganisms (27). This is because microbes with catalase are able to break down both host and microbial hydrogen peroxide and escape

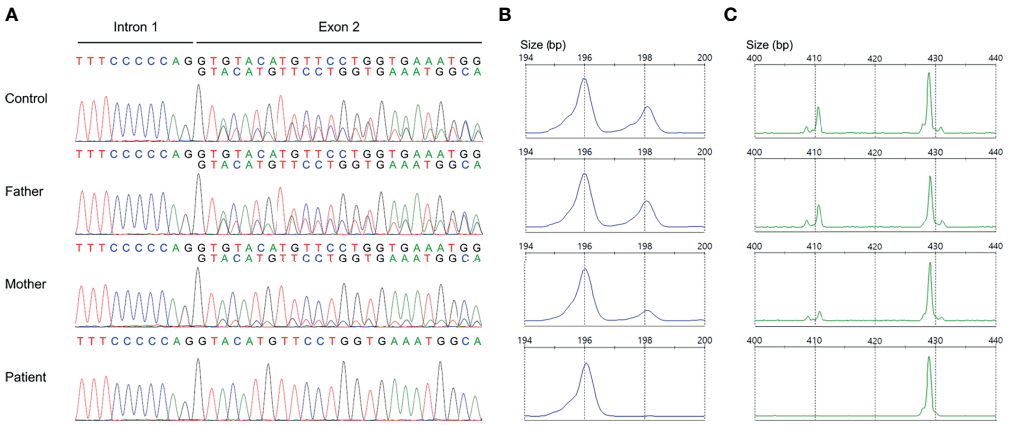

FIGURE 3 | Validation of GT-deletion (ΔGT) in *NCF1*. **(A)** Coamplification of *NCF1* and its pseudogenes at intron 1/exon 2. Overlapping peaks of GTGT-containing *NCF1* and ΔGT-containing pseudogenes were observed in the control and parents. The patient only had a product of *NCF1* pseudogenes, indicating a ΔGT in *NCF1* (NM_000265.7: exon 2: c.75_76delGT: p.Tyr26Hisfs*26). **(B)** ΔGT gene scan. The fragments of *NCF1* and its pseudogenes were depicted by the 198-bp peak and 196-bp peak respectively. A normal ratio of 2:4 was identified in the control and father, reflecting two copies of *NCF1* per four copies of pseudogenes. Meanwhile, the mother had a ratio of 1:5, signifying the carrier of ΔGT in *NCF1*. Only a single peak of *NCF1* pseudogenes was observed in the patient. **(C)** 20-bp gene scan. The 411-bp-peak and 429-bp-peak represented the fragments of *NCF1* and its pseudogenes respectively. Results from 20-bp gene scan corroborated the ratios from ΔGT gene scan. The patient was confirmed to have a biallelic loss of *NCF1*, in which one allele was included in the Williams-Beuren syndrome (WBS) deletion while another allele harbors a ΔGT. bp, base pair.

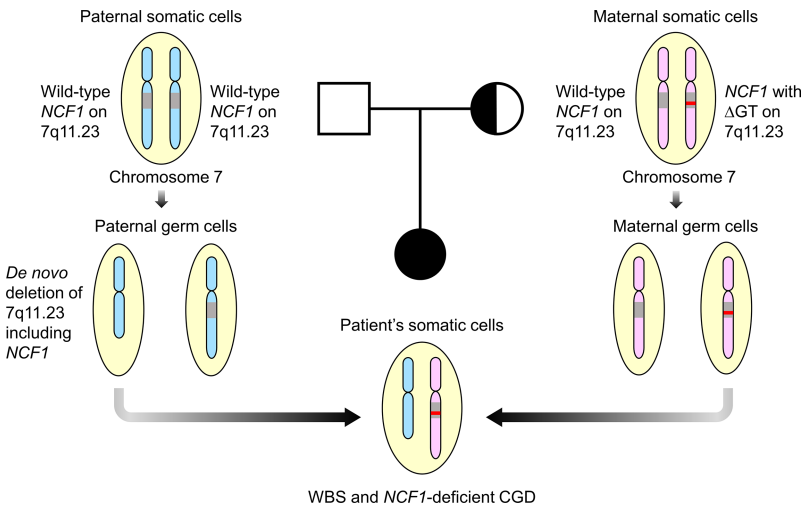

FIGURE 4 | Familial segregation analysis. The patient was postulated to inherit a paternal allele with *de novo* 7q11.23 deletion involving *NCF1* and a maternal allele with ΔGT-containing *NCF1*, resulting in Williams-Beuren syndrome (WBS) and *NCF1*-deficient chronic granulomatous disease (CGD). Both parents were well with no features of WBS and CGD. Square symbol indicates male, half-filled circle indicates female carrier of *NCF1* with ΔGT and filled circle indicates female affected by WBS and CGD. The diagram was not drawn to scale. ΔGT, GT-deletion; WBS, Williams-Beuren syndrome; CGD, chronic granulomatous disease.

from the oxidative killing. On the contrary, catalase-negative microorganisms seldom caused infections in CGD patients as they were killed by their self-produced hydrogen peroxide. Interestingly, we detected the growth of catalase-negative *Streptococcus* sp. in our patient in addition to catalase-positive bacteria, i.e., *Escherichia coli* and *Salmonella typhi*. The pathogenicity of *Streptococci* in CGD can be explained by some strains that produced no hydrogen peroxide (33, 34). Hence, the possibility of getting infected by catalase-negative microbes should be taken into consideration despite being rare in CGD patients.

Misalignment of segmental duplications on chromosome 7q11.23 mediates nonallelic homologous recombination during meiosis, resulting in WBS deletion (35). Cases with a 1.5Mb

deletion are more common than those with a larger deletion of 1.8Mb (36). Diagnosing WBS using fluorescence *in situ* hybridization with an *ELN*-targeting probe hampers the detection of deletion size (37). This limitation was then overcome by chromosomal microarray and multiplex ligation-dependent probe amplification (38, 39). Alternatively, we used read depth data from WES to call CNVs, revealing a heterozygous deletion of 1.53Mb on chromosome 7q11.23. The deletion breakpoints were predicted within *NSUN5* and *GTF2IRD2*, affecting 27 genes, including *ELN* and *NCF1*. Phenotypic consequences were identified in some of the deleted genes. As reported by Curran et al., haploinsufficiency of *ELN* was responsible for the pathogenesis of aortic stenosis (40). This heart abnormality was noted in our patient, whom had single copy of *ELN*. Arterial stiffening, possibly due to elastin insufficiency, may increase the risk for cardiovascular events (41, 42). Contrastingly, hemizygosity for *NCF1* generated lower oxidative stress, serving as a protective factor for hypertension (43). Biallelic pathogenic mutations in *NCF1* will lead to autosomal recessive CGD (20). Stenton et al. recently revealed a causal link between homozygous mutations in *DNAJC30* and autosomal recessive Leber's hereditary optic neuropathy in humans (44). This feature has yet to be reported in WBS patients. Understanding the potential impacts of genetic variants help to predict the disease progression and tailor a proper therapeutic treatment.

Dual molecular diagnosis caused by two disease loci within a patient was less widely recognized before the adoption of NGS. According to a retrospective analysis of exome by Smith et al., patients with clinically complex phenotypes were more likely to have multiple genetic diagnoses (45). This finding suggests the need for reevaluation of existing NGS data and additional laboratory screening in patients with phenotypic complexity. Our study described the first case from Southeast Asia, whom was diagnosed with WBS and *NCF1*-deficient CGD using WES. Until now, only six patients were reported with similar dual diagnosis (46–50). Despite *NCF1* hemizygosity conferred a lower risk for hypertension, Stasia et al. reported one *NCF1*-deficient patient suffering from high blood pressure (49). Hence, regular medical evaluation is crucial to detect the unexpected medical conditions and initiate a timely regimen.

There are several limitations in this study. Firstly, WES impedes the detection of true positive variants in genes with pseudogenes and highly repetitive sequence due to poor mapping in these regions. Although WES may not be an ideal approach for mutation screening in *NCF1*, it is useful for the identification of exonic and splicing variants in other CGD genes. Additional genetic testing, particularly WGS is required to detect disease-causing deep intronic variants that will be missed by WES. Secondly, uneven coverage resulted from amplification bias in WES may reduce the sensitivity and accuracy of CNV detection. Wide coverage of WGS made it more superior to detect CNVs that affect both coding and noncoding regions. Chromosomal microarray and multiplex ligation-dependent probe amplification can be used to validate CNVs identified from NGS data.

CONCLUSION

Our study revealed a case with dual diagnosis of WBS and *NCF1*-deficient CGD. Blended phenotypes of two genetic conditions can get misinterpreted as atypical presentation of a single disease, resulting in misdiagnosis or underdiagnosis. The use of NGS has facilitated the elucidation of multiple molecular diagnoses caused by multilocus genomic variations. Still, NGS has a low variant detection rate in genes with homologous pseudogenes, which can be complemented by Sanger sequencing. This study demonstrated the capacity of WES to detect both germline short variants and CNVs that may provide insights into pathogenesis and optimal treatment options.

DATA AVAILABILITY STATEMENT

The dataset presented in this study can be found in online repository. The name of the repository and accession number are as follows: https://www.ncbi.nlm.nih.gov/, PRJNA763322.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Research and Ethics Committee, Ministry of Health Malaysia. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AMR and SBM designed the study framework and supervised the study. MYC performed the experiments and data analysis. MYC and AMR drafted the manuscript. RRPR recruited the patient, provided clinical treatments and contributed critical views to the study. All co-authors critically reviewed and approved the final version of the manuscript.

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IL-21 Rescues the Defect of IL-10-Producing Regulatory B Cells and Improves Allergic Asthma in DOCK8 Deficient Mice

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Mutations in human DOCK8 cause a combined immunodeficiency syndrome characterized by allergic diseases such as asthma and food allergy. However, the underlying mechanism is unclear. Regulatory B (Breg) cells that produce IL-10 exert potent immunosuppressive functions in patients with allergic and autoimmune disorders. DOCK8-deficient B cells show diminished responses to TLR9 signaling, suggesting a possible defect in IL-10-producing Breg cells in those with DOCK8 deficiency, which may contribute to allergies. Here, we isolated peripheral blood mononuclear cells from DOCK8-deficient patients and generated a Dock8 KO mouse model to study the effect of DOCK8 deficiency on Breg cells. DOCK8-deficient patients and Dock8 KO mice harbored quantitative and qualitative defects in IL-10-producing Breg cells; these defects were caused by abnormal Dock8$^{-/-}$ CD4^{+} T cells. We found that recombinant murine (rm) IL-21 restored the function of Bregs both *in vitro* and in Dock8 KO mice, leading to reduced inflammatory cell infiltration of the lungs in a murine asthma model. Overall, the results provide new insight into the potential design of Breg-based or IL-21-based therapeutic strategies for allergic diseases, including asthma associated with DOCK8 deficiency.

Keywords: regulatory B cells, IL-10, DOCK8 deficiency, asthma, IL-21

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INTODUCTION

Mutations in dedicator of cytokinesis 8 (DOCK8) are the major cause of autosomal recessive hyper-IgE syndromes (HIES), which are characterized by combined immunodeficiency and elevated serum IgE levels (1). Patients with HIES caused by DOCK8 deficiency are more susceptible to developing allergic diseases (e.g., asthma, food allergies, and atopic dermatitis) than those with HIES caused by STAT3 mutations (2). Recent work identified some of the mechanisms underlying the high incidence of allergy in DOCK8 deficiency. For example, Dock8$^{-/-}$ IL-13^{hi} IL-4^{hi} IL-5^{hi} IL-21^{lo} follicular helper T (Tfh)13 cells are associated with production of high-affinity IgE antibodies (3), and migration-induced cell shattering causes a type 2-biased helper T cell response (4). However, the role of DOCK8-deficient B cells in the pathogenesis of allergic diseases remains unclear.

DOCK8 functions as a guanine nucleotide exchange factor that is important for actin cytoskeleton rearrangement and optimal STAT3 phosphorylation; it also serves as an adaptor molecule for TLR9-MYD88 signaling in B cells (5, 6). Loss-of-function mutations in DOCK8 contribute to impairment of B cell function and of long-lived memory responses (7).

B cells play an important role in the pathogenesis of allergic diseases, in particular by secreting IgE. However, regulatory B (Breg) cells in humans and mice are defined as B cells with immunosuppressive capacity associated with secretion of anti-inflammatory cytokines such as TGF-β, IL-35, and, particularly, IL-10 (8–11). Several Breg subsets exert immunoregulatory functions in allergic and inflammatory diseases by secreting IL-10; these cells, such as mouse CD1d^{hi}CD5^{+} B cells (termed B10 cells) (12), CD21^{+}CD23^{-} marginal zone (MZ) B cells (13), and human CD19^{+}CD24^{hi}CD27^{+} memory B cells (14), and CD19^{+}CD24^{hi}CD38^{hi} immature transitional B cells (15) share partially overlapping phenotypes. IL-10-producing Breg cells have direct and indirect suppressive effects on proliferation and cytokine production by effector T cells. With respect to the signaling mechanisms underlying development of IL-10-producing Breg cells, previous studies report that BCR-derived signals initiate acquisition of regulatory B10-like competence. Then, LPS-induced TLR4 and TLR9 signaling facilitates a transcriptionally active conformation of the *IL10* gene in pro-B10 cells (16, 17); also, activation of STAT3 is required for TLR-induced IL-10 production by B cells (18, 19). In addition, IL-21- and CD40-dependent cognate interactions with T cells are required to generate fully functional mouse B10 cells (20). DOCK8-deficient B cells show diminished responses to TLR9 signaling, suggesting a possible defect in IL-10-producing Breg cells in those with DOCK8 deficiency, which may contribute to allergies.

IL-21 is a type I cytokine produced mainly by activated CD4^{+} T cells and natural killer T (NKT) cells (21, 22). Upon binding to its receptors (IL-21R and a common receptor γ chain), IL-21 activates the Janus family tyrosine kinases members JAK1 and JAK3, with subsequent phosphorylation of STAT3 and STAT1 (23). For functionality, IL-21 mediates maturation of B cells, normal development of T follicular helper cells, and differentiation of Th17 cells (24).

In this study, we generated Dock8 KO mice using the TALEN technique; these mice harbor a frameshift mutation in the first exon of *Dock8*, which mirrors human disease because most of the DOCK8-deficiency in patients is caused by the frame shift or gene deletion in different exons of the DOCK8 gene rather than by a point mutation. We found that DOCK8-deficient patients and Dock8 KO mice harbor both quantitative and qualitative defects in IL-10-producing Breg cells due to abnormalities in Dock8$^{-/-}$ CD4^{+} T cell populations. We also found that IL-21 rescued the function of Bregs in Dock8 KO mice and alleviated inflammatory infiltration in a murine asthma model.

MATERIALS AND METHOD

Patients

Three Chinese patients with mutations in the DOCK8 gene were enrolled in the study. All patients were admitted to the Children's Hospital of Chongqing Medical University. Diagnosis of the patients was described previously (25). Three age-matched subjects were enrolled as healthy controls (HCs). Informed consent to participate in the study was provided by the patients' families, and the study was approved by the Medical Ethics Committee of Children's Hospital of Chongqing Medical University.

Mice

Dock8 KO mice were generated using the TALEN technique (Shanghai Biomodel Organism Science & Technology Development Co., Ltd). The first exon of *Dock8* was chosen for TALEN-induced mutagenesis; absence of a 45 bp sequence from the coding frame of exon 1 introduced a reading frame shift into the Dock8 gene (26). Dock8 was genotyped by PCR using the following primer pair: sense, 5'- GGGGGATCCCCTGC GGCCGGCGACTCTGA-3', and antisense, 5'- GGGGAATTC GAAGCGGGGAAGGCAATGATGACA-3'. PCR products amplified from F0 generation mouse tail tissue were purified, cloned, and sequenced to identify positive founder mice with the Dock8 protein harboring the frame shift. Positive F0 generation mice were crossed with C57BL/6J mice and the genotype of the offspring was confirmed by PCR, cloning, and sequencing. CD4 KO and CD45.1^{+} C57BL/6 mice were obtained from the Jackson Laboratory. C57BL/6 mice were purchased from the Laboratory Animal Center, Chongqing Medical University. All mice were aged 6 to 10 weeks at the time of the experiments and were housed in specific pathogen-free animal facilities. Data were obtained from three or more mice per group. All animal experiments were reviewed and approved by the Institutional Animal Care and Usage Committee of Children's Hospital of Chongqing Medical University.

Flow Cytometry and Phosphorylation Analyses

Heparinized blood was obtained from patients and from age-matched HC subjects. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation and cell numbers were counted in a hemocytometer. Flow cytometry was performed using a FACSCanto II cytometer (BD Biosciences, San Jose, Calif). Briefly, PBMCs were stained with anti-

human CD19-APC, anti-human CD24-PE, anti-human CD27-BV450, anti-human CD38-PerCP-cy5.5, or anti-human IgD-BV510. Mononuclear cells isolated from the spleen of DOCK8 KO and WT mice were stained with the following antibodies: anti-CD19 FITC, anti-CD5 PE, and anti-CD1d APC ($CD1d^{hi}CD5^{+}$ B cells); anti-CD19 FITC, anti-CD23 PE, anti-CD21 APC, and anti-CD24 Percp-cy5.5 (T2-MZP cells) (all antibodies were from BioLegend, CA). Mononuclear cells isolated from spleen cells of chimera mice were stained with anti-CD45.1 BV510, CD45.2 FITC, anti-CD19 APC, and anti-IL-10 PE (BioLegend, CA). Mononuclear cells isolated from the spleen of CD4 KO mice were stained with anti-CD19 FITC or APC, anti-CD5 PE, anti-CD1d APC, anti-CD23 PE, anti-CD21 APC, anti-CD24 Percp-cy5.5, or anti-IL-10 PE (BioLegend, CA). To detect phosphorylation, single-cell suspensions of splenocytes were stimulated for 3 h with lipopolysaccharide (LPS; 10 μg/mL, Sigma, St. Louis) or for 30 min with rmIL-21 (100 ng/mL; R&D Systems). Cells were then fixed and permeabilized with BD Phosflow Lyse/Fix Buffer and Perm Buffer III (BD Biosciences), respectively. Finally, cells were stained with anti-B220 FITC, anti-CD5 BV421, anti-CD1d APC, or anti-pY727 PE. All data were analyzed using FlowJo software.

B Cell Stimulation

PBMCs isolated from patients or HCs were resuspended (at 2×10^6 cells/mL) in 48-well flat-bottom plates in culture medium and stimulated for 7 h with LPS (10 μg/mL; Sigma, St. Louis), phorbol 12-myristate 13-acetate (PMA, 20 ng/mL; Sigma, St. Louis), ionomycin (1 μg/mL; Sigma, St. Louis), and brefeldin A (BFA, 1×solution/mL; BioLegend, CA) before staining and flow cytometry analysis. Next, the cells were harvested and washed twice with PBS. Single-cell suspensions were then stained for 20 min on ice with predetermined optimal concentrations of anti-CD19 APC (BioLegend, CA). Stained cells were washed twice with PBS before fixation and permeabilization in Fixation and Permeabilization Buffer (BioLegend, CA). Finally, cells were stained for 30 min with anti-IL-10 PE (BioLegend, CA). To detect B10 cells in mice, single-cell suspensions of splenocytes were stimulated for 5 h with LPS, PMA (50 ng/mL), ionomycin (500 ng/mL), and BFA, followed by staining with anti-CD19 APC (BioLegend, CA) and anti-IL-10 PE (BioLegend, CA). For co-culture experiments, splenic B cells were purified using an EasySep™ Mouse B Cell Isolation Kit (STEMCELL Technologies, Canada). The cells (2×10^6 cells/mL) were then incubated for 48 h with mouse rmIL-21 (100 ng/mL, R&D Systems). After culture for 48 h, the IL-10 concentration in the supernatant was measured in an ELISA (BioLegend, CA), and B10 cell numbers were measured by flow cytometry analysis, as described above.

Generation of Bone Marrow Chimeras

Bone marrow was collected from $Dock8^{-/-}$ ($CD45.2^{+}$) mice and from C57BL/6 wild-type ($CD45.1^{+}$) mice. For each chimera, $CD45.2^{+}$ wild-type or Dock8 KO bone marrow cells plus $CD45.1^{+}$ bone marrow cells (4×10^6 cells in a 1:1 mixture) were transferred intravenously into lethally-irradiated (two doses of 550 rads each) wild-type $CD45.1^{+}$ recipients. Recipient mice were allowed 8 weeks to reconstitute prior to challenge with ovalbumin (OVA).

Generation of OVA-Induced Allergic Asthma Model Mice and Nasal Administration of Recombinant IL-21

OVA-induced allergic asthma was elicited by sensitization with chicken OVA (5 μg, intraperitoneally (i.p.); Sigma-Aldrich) emulsified in 200 μl Imject Alum (Thermo Fisher Scientific) on Day 0, followed by two oropharyngeal aspiration challenges (on Days 14 and 21) with 1.5% OVA dissolved in 50 μl PBS. Control mice received only PBS. Mice were harvested 72 h after the second challenge (27). Some mice received 20 ng of rmIL-21 (R&D Systems) into the nostrils (daily on Days 15–17, 18–20, or 15–20). This 3 day protocol was decided by conducting preliminary time-course experiments. The dose of rmIL-21 was obtained from a previous study (28).

Adoptive Cell Transfer

Naïve splenic $CD4^{+}$ T cells isolated from Dock8 KO or wild-type mice were purified with the EasySep™ Mouse Naïve $CD4^{+}$ T Cell Isolation Kit (STEMCELL Technologies, Canada). Next, 5×10^6 naïve $CD4^{+}$ T cells were adoptively transferred into CD4 KO mice *via* intravenous injection 1 day before immunization with OVA.

TABLE 1 | Clinical features of DOCK8-deficient patients.

Features	P1/Male	P2/Female	P3/Male
Age at onset	6 y	1 y 7 m	3 y
Age at diagnosis	14 y	5 y 8 m	9 y
DOCK8 mutation	Exon11 homozygous deletion + Exon 12–33 heterozygous deletion	Exon2 homozygous deletion + Exon1, 3–39 heterozygous deletion	c.1278-1279delTG, p.V427fsX435
DOCK8 protein expression	–	–	–
Atopy	Eczema, asthma	Eczema,	Eczema, food allergy
Immunodeficiency	Respiratory infection, otitis media, sinusitis, stomatitis	Respiratory infection, otitis media, stomatitis	Respiratory infection, otitis media, stomatitis
Autoimmune disease	–	–	Autoimmune hemolytic anemia
Malignancy	–	–	–
Etiology of infections	HSV	EBV	EBV
Molluscum contagiosum	+	+	+

HSV, herpes simplex virus; EBV, Epstein–Barr virus. ; –, negtive; +, positive.

OVA immunization was performed as described above. 100 µl blood was taken from the tail vein of CD4KO mice on day 7, day14 and day 21 to detect the number of $CD4^+$ T cells. Recipient mice were analyzed on Day 24 post-OVA immunization.

Histopathological Analysis

Lung tissue was harvested and fixed for 24 h in 10% formalin and then embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin. The degree of airway infiltration by inflammatory cells was scored by two independent investigators by double-blind screening. Peri-bronchiole and peri-vascular inflammation were evaluated using a scoring system of 0–4, where 0 represents no cells; 1, a few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; and 4, a ring of inflammatory cells >4 cells deep.

Statistical Analysis

GraphPad Prism 5 software was used for statistical analyses. Results are expressed as the mean ± SEM. The significance of differences between groups was determined using a two tailed unpaired Student's *t* test or ANOVA. A P value <0.05 was deemed statistically significant.

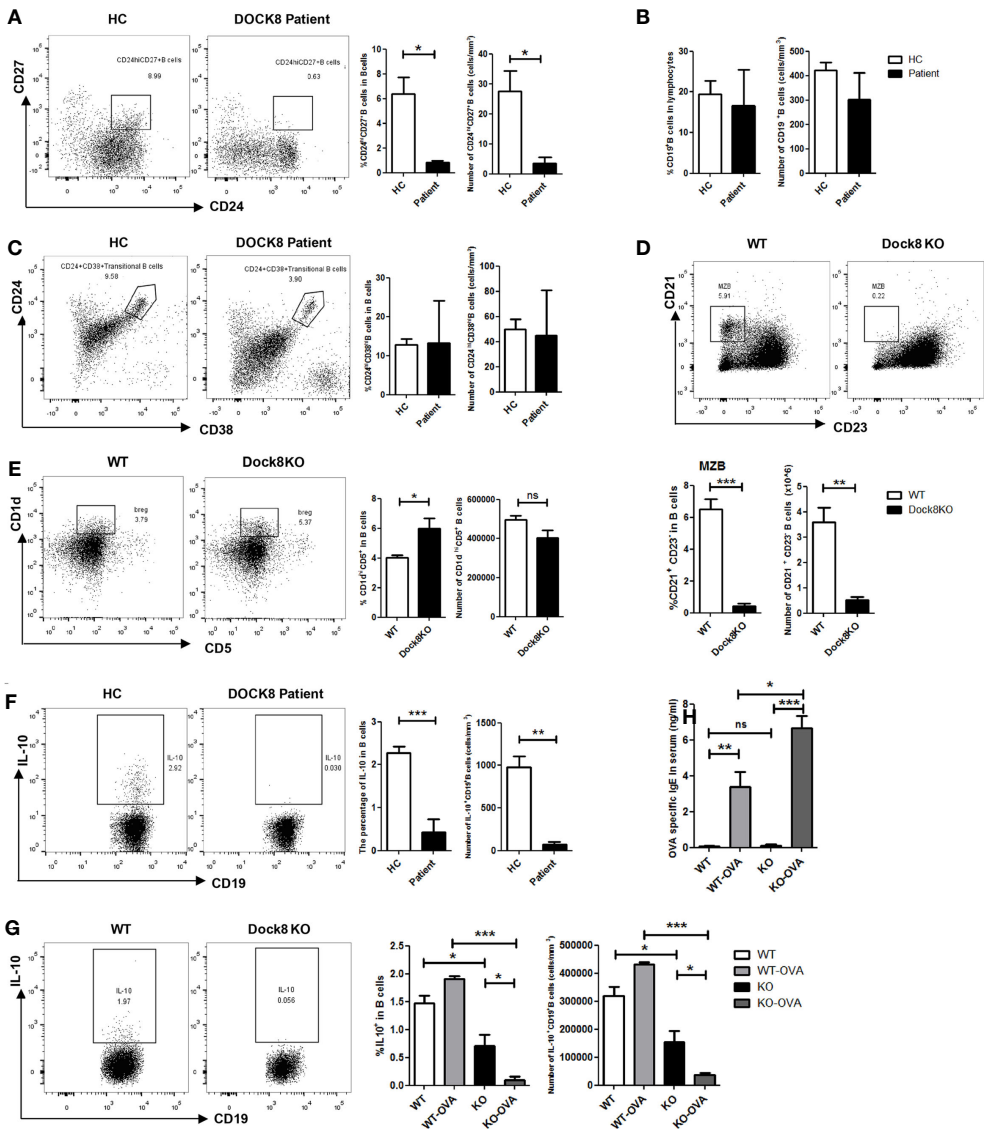

FIGURE 1 | Reduced percentage of IL-10-producing Breg cells in DOCK8-deficient patients and Dock8 KO mice. **(A)** Percentage and number of $CD19^+CD24^{hi}CD27^+$ B cells, **(B)** $CD19^+$ B cells, and **(C)** $CD19^+CD24^{hi}CD38^{hi}$ B cells in HCs and DOCK8-deficient patients (n = 3 for both). **(D)** $CD21^+CD23^-$ marginal zone B cells and **(E)** $CD5^+CD1d^{hi}$ B cell populations in the spleen of wild-type and Dock8 KO mice (n = 4 for both). **(F)** The percentage of IL-10^+CD19^+ Breg cells in HCs and DOCK8-deficient patients (n = 3 for both). **(G)** Percentage of IL-10^+CD19^+ Breg cells in the spleen of wild-type and Dock8 KO mice immunized (or not) with OVA (n = 4 for both). **(H)** OVA-specific IgE in serum of Dock8 KO mice immunized with OVA was measured by ELISA. *P < 0.05, **P < 0.01, and ***P < 0.001, and ns is not significant (Student's *t* test). Data are representative of three independent experiments.

RESULTS

Breg Subsets Are Present in DOCK8-Deficient Patients and Dock8 KO Mice, but Are Not Capable of Producing IL-10

To investigate the effects of DOCK8 deficiency on homeostasis of Breg cells in humans, we isolated PBMCs from three patients (aged 5–14 years) with confirmed DOCK8 deficiency (**Table 1**). First, we examined Breg subsets. We found that the percentage and number of $CD19^+CD24^{hi}CD27^+$ memory B cells within the PBMC population was lower in patients than in age-matched HCs (**Figure 1A**), whereas the total B cell (**Figure 1B**) and $CD19^+CD24^{hi}CD38^{hi}$ immature transitional B cell populations (**Figure 1C**), which harbor IL-10-producing Breg cells, were normal. In Dock8 KO mice, analysis of splenic B cells revealed a near absence of $CD21^+CD23^-$ marginal zone B cells (**Figure 1D**) but a higher frequency of $CD1d^{hi}CD5^+$ B10 cells (**Figure 1E**) than in controls; these cell populations also harbor IL-10-producing Breg cells.

Next, we examined whether DOCK8 expression affects the function of Breg cells in DOCK8-deficient patients and Dock8 KO mice. PBMCs from patients and HCs were stimulated for 7 h with LPS plus PMA, ionomycin, and BFA to determine whether the absence of DOCK8 affects IL-10 production by B cells. The results showed that $IL\text{-}10^+$ B cell numbers in patients were significantly lower than those in HCs; indeed, they were almost absent from patients (**Figure 1F**). The percentage and number of $IL\text{-}10^+$ B cells in Dock8 KO mice were also lower than those in wild-type mice, especially after immunization with OVA (**Figure 1G**). Previously, we reported that, compared with wild-type mice, Dock8 KO mice show increased serum IgE levels and develop significant airway inflammatory infiltration and airway hyper-responsiveness in the OVA-induced allergic asthma model [20], a finding that is consistent with the data presented above; the serum level of OVA-specific IgE in Dock8 KO mice was also increased (**Figure 1H**).

DOCK8 Deficiency Causes a Partial Intrinsic Defect in Breg Cells

To investigate whether the functional defect in Breg cells from DOCK8 KO mice is intrinsic to these cells, we transferred $CD45.2^+$ wild-type or DOCK8 KO bone marrow cells plus $CD45.1^+$ bone marrow cells (4×10^6 cells in a 1:1 mixture) into lethally-irradiated (two doses of 550 rads each) wild-type $CD45.1^+$ recipients. Recipient mice were allowed 8 weeks to reconstitute before being challenged with OVA. We found that the percentage of $CD45.2^+IL\text{-}10^+$ B cells (**Figures 2A, B**) and $CD45.2^+CXCR5^+$ Tfh cells (**Supplemental Figure 1**) in the spleens of immunized chimeric mice harboring Dock8-deficient bone marrow cells decreased. However, B cells in the mixed chimeras retained some IL-10-producing functions (the frequency of $IL\text{-}10^+$ B cells was about 0.5%, whereas that in OVA-immunized Dock8 KO mice was almost

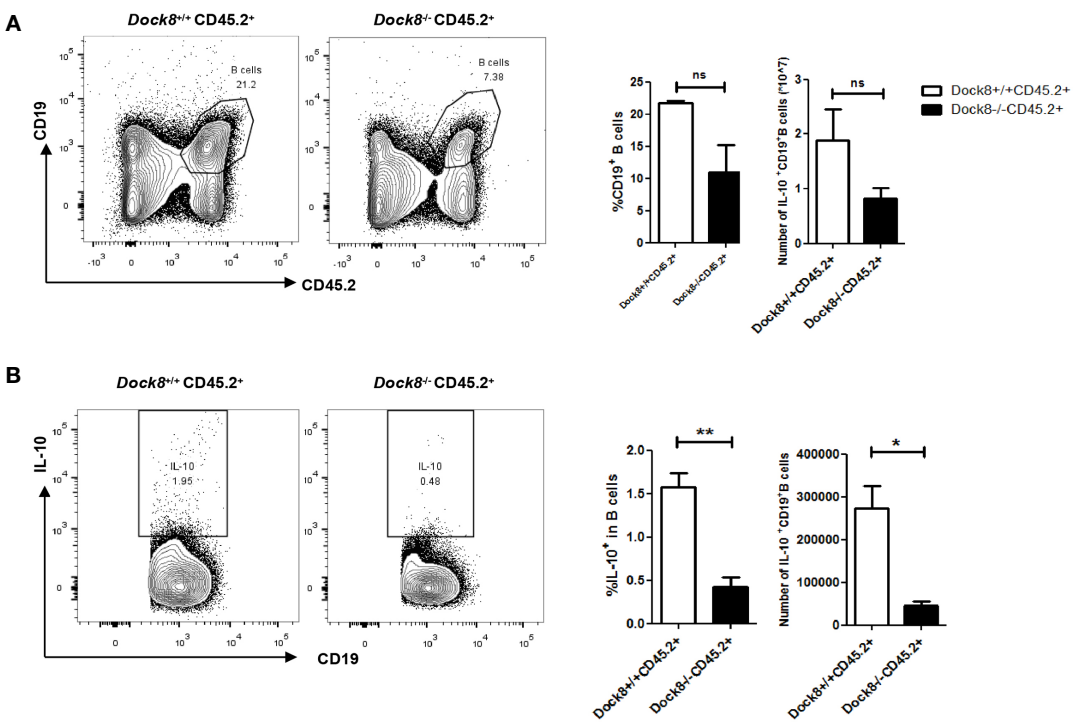

FIGURE 2 | Breg defect in Dock8 deficiency is not entirely B cell intrinsic. **(A, B)** $CD45.2^+$ wild-type or Dock8 KO bone marrow cells were transferred intravenously into lethally-irradiated $CD45.1^+$ wild-type recipients (n = 3 for both) prior to immunization with OVA. Flow cytometry analysis of $CD45.2^+CD19^+$ B cells and $CD45.2^+$ $CD19^+IL\text{-}10^+$ B cells in the splenocyte population from $CD45.1^+$ chimeric mice. *P < 0.05, **P < 0.01, and ns is not significant (Student's *t* test). Data are representative of two experiments.

0% (see **Figure 1G**), suggesting that IL-10 secretion by Dock8-deficient B cells may also be affected by other cells. These results indicate that the Breg defect in Dock8 deficiency is not entirely B cell-intrinsic.

DOCK8$^{-/-}$ CD4$^+$ T Cells Impair IL-10 Production by B Cells

Breg cells require cognate interactions with IL-21-producing CD4$^+$ T cells to secrete IL-10 *in vivo* (20), and abnormalities in CXCR5$^+$CD4$^+$ Tfh cells associated with DOCK8 deficiency has been described previously (29). Therefore, we performed adoptive transfer experiments to confirm the effect of Dock8$^{-/-}$ CD4$^+$ T cells on IL-10 production by B cells. CD4 KO mice received wild-type or Dock8 KO splenic CD4$^+$ naïve T cells, followed by challenge with OVA. The adoptively transferred CD4$^+$ T cells in the blood of CD4 KO mice were detected on day 7, day14 and day21 (**Supplemental Figure 2**). At 24 days post-immunization with OVA, we analyzed the IL-10$^+$ Breg cell population in CD4 KO recipient mice (**Figure 3A**). The percentage of IL-10$^+$ Breg cells in CD4 KO mice receiving Dock8 KO CD4$^+$ naïve T cells was lower than that in mice receiving wild-type CD4$^+$ naïve T cells (**Figure 3B**). There was no significant difference between two groups with respect to the percentage of CD1d^{hi}CD5$^+$ B cells or MZB cells (**Figure 3C**). Thus, Dock8 KO CD4$^+$ naïve T cells do not affect the percentages of Breg subsets in recipient mice. These results suggest that loss of DOCK8 from CD4$^+$ T cells attenuates IL-10 production by B cells *in vivo*.

Supplementation With IL-21 Restores IL-10 Production by B Cells From DOCK8 KO Mice Both *In Vitro* and *In Vivo*

To confirm whether reduced IL-21 secretion by CD4$^+$ T cells under conditions of Dock8 deficiency causes the functional defect in Breg cells, we co-cultured purified splenic B cells (2×10^6) from wild-type or Dock8 KO mice with IL-21. After culture for 48 h, the IL-10 concentration in the supernatant was measured in an ELISA. Whereas the percentage of IL-10$^+$ B cells in Dock8 KO mice was comparable with that in WT mice (**Figure 4A**), the concentration of IL-10 was higher (**Figure 4B**).

Next, to further examine the effect of IL-21 on IL-10 production by B cells *in vivo*, we exposed wild-type or Dock8 KO mice to intranasal rmIL-21 for 3 or 6 days. Surprisingly, the percentage of IL-10$^+$ B cells in the spleen of Dock8 KO mice was almost comparable with that in wild-type mice (**Figure 4C**). Increased

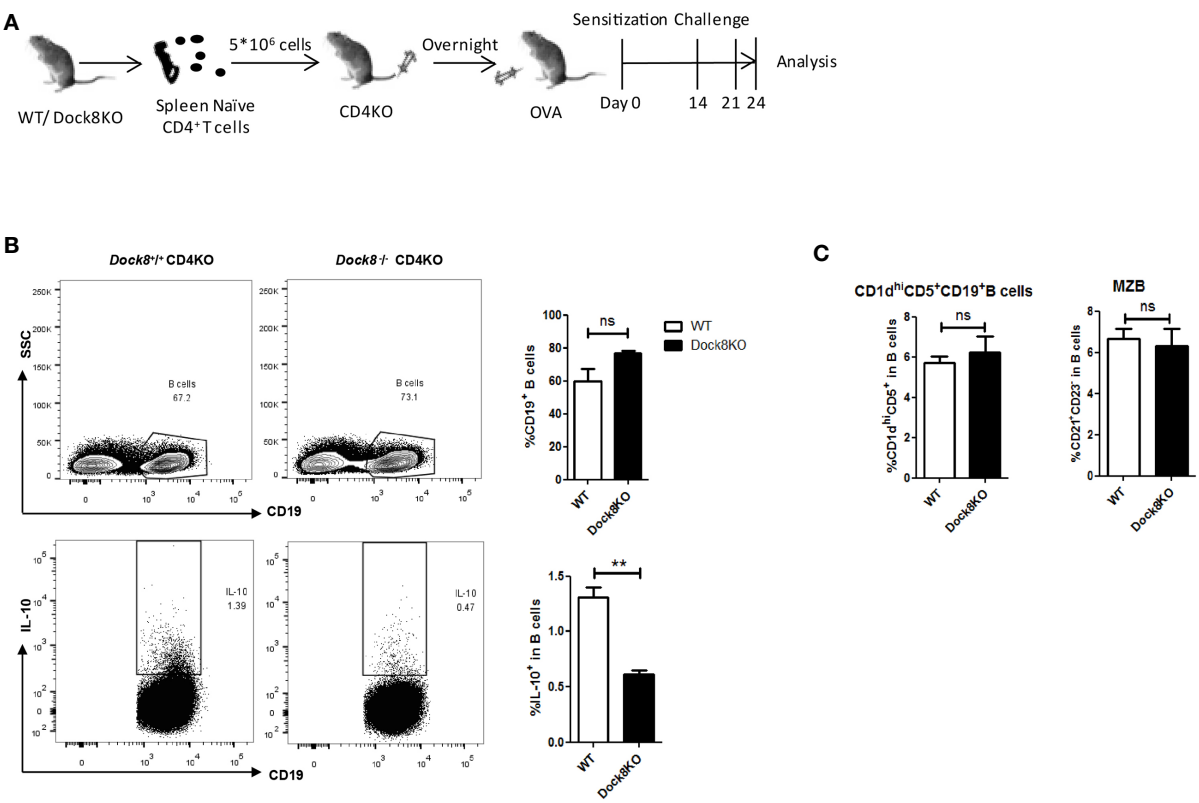

FIGURE 3 | Dock8$^{-/-}$ CD4$^+$ T cell impairs IL-10 production by B cells. **(A)** Splenic CD4$^+$ naïve T cells from wild-type (n = 4) or Dock8 KO mice (n = 4) were transferred into CD4 KO mice 1 day before immunization with OVA. Mice were sensitized with 5 μg OVA intraperitoneally on Day 0, followed by two oropharyngeal aspiration challenges (on Days 14 and 21). Mice were harvested 72 h after the second challenge. **(B)** The percentage of CD19$^+$IL-10$^+$ B cells in CD4 KO mice receiving Dock8$^{-/-}$ CD4$^+$ naïve T cells was lower than that in mice receiving wild-type CD4$^+$ naïve T cells. **(C)** Dock8$^{-/-}$ CD4$^+$ T cells have no effect on the percentage of different Breg subtypes. **P < 0.01, and ns is not significant (Student's *t* test). Data are representative of two independent experiments.

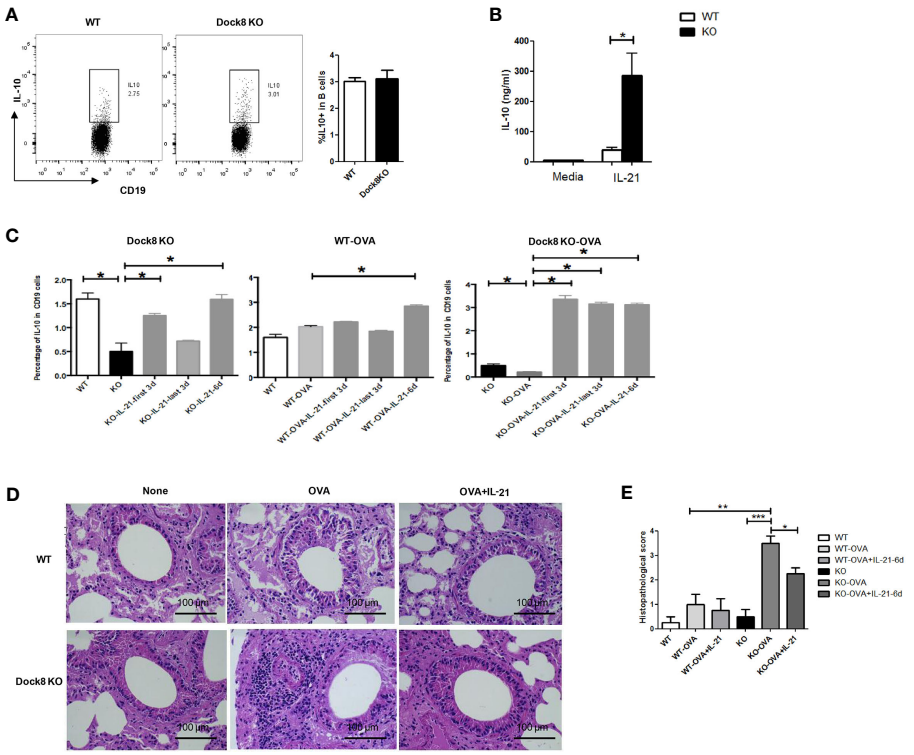

FIGURE 4 | Exogenous IL-21 rescues IL-10 production by Breg cells in Dock8 KO mice both *in vitro* and *in vivo*. **(A)** Splenic B cells isolated from wild-type (n = 3) or Dock8 KO mice (n = 3) were co-cultured with IL-21 for 48 hours and the percentage of $CD19^{+}IL\text{-}10^{+}$ B cells was analyzed by FCM. **(B)** The IL-10 concentration in the supernatant was measured in an ELISA. **(C)** The percentage of $CD19^{+}IL\text{-}10^{+}$ B cells in wild-type (n = 3) and Dock8 KO mice (n = 3), which were sensitized with 5 μg OVA on Day 0, followed by two challenges (on Days 14 and 21). Finally, 20 ng of rmIL-21 was administered daily (for 3 or 6 days; Days 15–17, 18–20, or 15–20) into the nostrils. Mice were harvested 72 h after the second challenge. **(D)** Representative images of H&E stained lung tissue from each genotype in the OVA-induced allergic asthma model."OVA+IL-21" images are from 6 days of IL-21 treatment. **(E)** Histopathological score for airway inflammation. *P < 0.05, **P < 0.01, ***P < 0.001, and ns is not significant (Student's *t* test for **(A, B)**, ANOVA for **(C–E)**. Data in **(A, B, D)** are representative of three independent experiments. Data in **(C)** are representative of two experiments.

production of IL-10 by B cells was more obvious in Dock8 KO mice sensitized with OVA. At the same time, treatment with recombinant IL-21 provided Dock8 KO mice with marked protection from OVA-induced airway inflammation, accompanied by alleviation of inflammatory infiltration (**Figures 4D, E**). Whereas treatment with rmIL-21 fully restored the percentage of $IL\text{-}10^{+}CD19^{+}$ B cells in the spleens of Dock8 KO mice to WT levels (**Figure 4C**), it only partially alleviated airway inflammation (**Figure 4E**), suggesting that other cells might also play a role. Taken together, these data suggest that IL-21 plays a critical role in the normal function of Breg cells under conditions of Dock8 deficiency, and that exogenous IL-21 rescues defective IL-10 production by B cells in Dock8 KO mice.

LPS-Driven, Not IL-21-Driven, STAT3 Phosphorylation Is Defective in Breg Cells From Dock8 KO Mice

Because STAT3 phosphorylation is required for LPS-induced IL-10 production by B cells (18, 19), we examined this phenomenon in B cells from Dock8 KO mice stimulated with LPS. We found that levels of STAT3 phosphorylation in Dock8 KO mice were lower than those in wild-type mice (**Figures 5A, B**). Next, we examined STAT3 phosphorylation in $CD1d^{hi}CD5^{+}$ B cells (B10 cells) from Dock8 KO mice after stimulation with LPS or IL-21. We found no significant increase in STAT3 phosphorylation in $Dock8^{-/-}$ $CD1d^{hi}CD5^{+}$ B cells after LPS stimulation (**Figure 5C**). By contrast, IL-21 caused comparable STAT3 phosphorylation in $CD1d^{hi}CD5^{+}$ B cells from Dock8 KO and wild-type mice (**Figures 5C, D**).

DISSCUSSION

Here, we present evidence that Dock8 deficiency impairs IL-10 production by Breg cells due to abnormalities in $Dock8^{-/-}$ IL-21-producing $CD4^{+}$ T cells. We also show that exogenous IL-21 rescues the function of Breg cells in Dock8 KO mice both *in vitro* and *in vivo*.

B cells play an important role in the pathogenesis of allergic diseases, in particular by secreting IgE. However, several

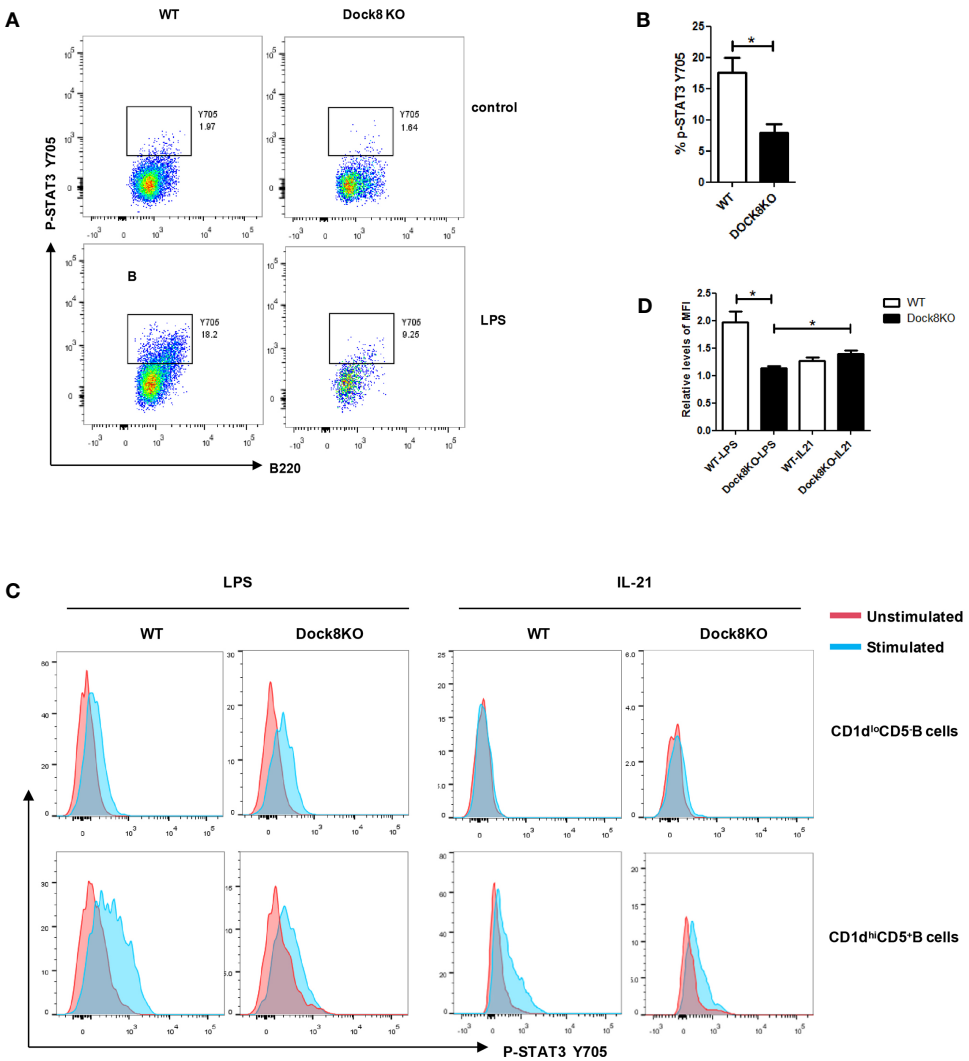

FIGURE 5 | LPS-induced STAT3 phosphorylation, but not IL-21-induced STAT3 phosphorylation, is defective in Breg cells from DOCK8 KO mice. **(A, B)** STAT3 phosphorylation on Y705 in splenic B cells from wild-type (n = 3) and Dock8 KO mice (n = 3) after 3 hours of LPS stimulation. **(C, D)** STAT3 phosphorylation in $CD5^{+}CD1d^{hi}$ B cells and $CD5^{-}CD1d^{lo}$ B cells after stimulation with LPS for 3 hours or rmIL-21 for 30 minutes (n = 3 for both).The red line is the unstimulated samples, the blue line is the stimulated samples. Mean fluorescence intensity (MFI) levels of IL-10 in unstimulated samples of respective groups were defined as 1. *P < 0.05 (Student's *t* test). Data in **(A)** are representative of three independent experiments, Data in **(C)** are representative of two independent experiments.

phenotypic subsets have been identified as Breg cells; these cells exert immunosuppressive functions in allergic and inflammatory diseases *via* release of IL-10 (30). In murine models of allergic airway disease, Breg deficiency is associated with increased serum IgE levels, increased secretion of type 2 cytokines, and increased eosinophilia (31, 32). Patients with allergic asthma and rhinitis show a decrease in the percentage of IL-10-secreting $CD19^{+}CD24^{hi}CD27^{+}$ Breg cells in response to LPS stimulation (14, 33). Injection of IL-10-producing $CD9^{+}$ Breg cells into asthmatic mice normalizes airway inflammation and lung function by inhibiting Th2- and Th17-driven inflammation (34).

In the present study, we used an OVA-induced allergic asthma model based on Dock8 KO mice, as previously described (27). Model mice showed severe reductions in the percentage of IL-10-producing Breg cells, increased serum OVA-specific IgE levels, and increased inflammatory infiltration compared with wild-type mice and Dock8 KO mice not exposed to OVA. DOCK8 deficiency is a HIES; therefore, it is associated with a high incidence of allergic disease. Indeed, 71% of patients have allergic manifestations and 30% develop asthma (35). Among the three patients examined in the present study, one patient had food allergies and one had asthma. Due to the small number of enrolled patients, we were unable to test whether the number of IL-10-producing Breg cells differed between patients with and without allergies. Further studies should clarify whether Breg cells are

involved in the onset of allergic disease under conditions of DOCK8 deficiency.

Our data also provide insight into the mechanism by which DOCK8 may cause a severe reduction in Breg cell numbers. IL-10 production by Breg cells requires LPS-induced TLR signaling (36). Nonetheless, IL-21- and CD40 dependent cognate interactions with T cells are also required for IL-10-producing Breg cells to optimally suppress inflammation and autoimmunity (20), and adding CD40L to the LPS cultures increased IL-10 producing B-cell frequencies in the health controls (**Supplemental Figure 3**). DOCK8-deficient patients may also have higher numbers of IL-10 producing B-cell if stimulated appropriately. All of these signals are instrumental to Breg expansion and function; thus dysfunction leads to impaired IL-10 production and susceptibility to inflammation and allergy. Because impaired expansion of $CXCR5^+CD4^+$ Tfh cells has been found in DOCK8 deficiency, we adoptively transferred $DOCK8^{-/-}$ $CD4^+$ T cells into CD4 KO mice and found that this impaired IL-10 production by B cells, which suggests that abnormalities of $Dock8^{-/-}CD4^+$ T cells affect the function of Breg cells. Subsequently, administration of rmIL-21 improved IL-10 production by Breg cells and ameliorated airway inflammation in Dock8 KO mice. All of these results confirm that IL-21-dependent interactions with T cells play a critical role in the normal function of Breg cells under conditions of DOCK8 deficiency. Reduced Breg cell numbers have also been observed in a variety of allergic diseases. Whether $IL\text{-}21^+$ Tfh cells are also involved in the defect of Breg cells remains to be explored.

As reported previously, TLR-driven STAT3 phosphorylation was defective in B cells from DOCK8-deficient patients, but IL-21 driven STAT3 phosphorylation in B cells from DOCK8-deficient patients was comparable with that in HCs (6). Our data suggest that LPS-driven, but not IL-21-driven, STAT3 phosphorylation is defective in Breg cells from Dock8 KO mice. Thus, it is possible that IL-21 restores the Breg defect in DOCK8 deficiency by inducing normal STAT3 phosphorylation.

Recently identified functions of DOCK8 explain why its loss might result in allergy; for example, generation of IgE-promoting $IL\text{-}13^+$ follicular helper T cells (3). These "Tfh13" cells produce IL-13 and IL-4, but downregulate production of IL-21. Consistent with this, our data suggest that defective $IL\text{-}21^+$ $CD4^+$ T cells might contribute to allergy in Dock8 KO mice, and that supplementation with IL-21 reduces airway inflammatory infiltration, as well as the number of serum IgE and IgE-producing B cells, in the OVA-induced allergic asthma model (27). Thus, IL-21 plays an important role in the pathogenesis of allergic asthma. However, mixed results have been reported regarding the impact of IL-21 in asthmatic mice; for example, studies suggest that IL-21 either promotes airway eosinophilia and type 2 immunity (21), or inhibits IgE production and decreases eosinophil recruitment into the airways (37). Tfh cells are the main cellular source of IL-21, which inhibits IgE class switch recombination in B cells by triggering STAT3 activation (38). Because IL-21 has a profound effect on IgE production, supplementation with IL-21 may rebalance the elevated IgE levels in patients with asthma. It is still not very clear why IL-21 signaling plays different roles in asthma; therefore, more studies are needed to provide definitive evidence.

In summary, we show here that DOCK8 regulates Breg function in both humans and mice. We propose that the absence of Bregs under conditions of DOCK8 deficiency contributes to allergy and chronic inflammation in these patients. Data from this study provide new insight into the potential design of Breg-based or IL-21-based therapeutic strategies for allergic diseases, including asthma in those with DOCK8 deficiency.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Children's Hospital of Chongqing Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Medical Ethics Committee of Children's Hospital of Chongqing Medical University.

AUTHOR CONTRIBUTIONS

JJ and XZ designed the study and wrote the manuscript. JJ, TQ, LiaZ, QL, JW, RD, LinZ, QZ, XL, and HW performed the experiments and analyzed the data. TQ and XZ followed-up the patients. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.695596/full#supplementary-material

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Reversion Mosaicism in Primary Immunodeficiency Diseases

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Reversion mosaicism has been reported in an increasing number of genetic disorders including primary immunodeficiency diseases. Several mechanisms can mediate somatic reversion of inherited mutations. Back mutations restore wild-type sequences, whereas second-site mutations result in compensatory changes. In addition, intragenic recombination, chromosomal deletions, and copy-neutral loss of heterozygosity have been demonstrated in mosaic individuals. Revertant cells that have regained wild-type function may be associated with milder disease phenotypes in some immunodeficient patients with reversion mosaicism. Revertant cells can also be responsible for immune dysregulation. Studies identifying a large variety of genetic changes in the same individual further support a frequent occurrence of reversion mosaicism in primary immunodeficiency diseases. This phenomenon also provides unique opportunities to evaluate the biological effects of restored gene expression in different cell lineages. In this paper, we review the recent findings of reversion mosaicism in primary immunodeficiency diseases and discuss its clinical implications.

Keywords: reversion, reversion mosaicism, somatic reversion, primary immunodeficiency diseases, selective advantage, gene therapy

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INTRODUCTION

Genetic mosaicism refers to an individual who has developed from a unique zygote but carries two or more cell types with different genotypes (1). This phenomenon is derived from postzygotic mutations, which can occur during embryonic development or during postnatal life (1). Based on the tissue distributions, genetic mosaicism is categorized into three types: gonadal mosaicism, gonosomal mosaicism, and somatic mosaicism (1). In gonadal and gonosomal mosaicism, postzygotic mutations affect the germline cells; therefore, mutant alleles can be transmitted to the offspring (1). However, in somatic mosaicism, postzygotic mutations occur only in somatic cells and may cause a disease related to the mutated gene, but they are not transmitted to the offspring (1). Reversion mosaicism refers to somatic mosaicism due to a reversion to normal of an inherited pathogenic mutation (2). In reversion mosaicism, reversion mutations partially or fully restore the effect of the primary disease-causing variant (2). The most common and simplest type of reversion is a true back mutation, which refers to the reversion of the germline mutation site to the wild-type sequence (3) (**Figure 1A**). As an alternative, a site-specific substitution is a nucleotide substitution at the specific germline mutation site, which restores the original amino acid sequence or the alternation to a less deleterious amino acid than in the original germline mutation (3) (**Figure 1B**). Reversion mutation also results from

second-site mutation, which occurs at a different site from the germline mutation but within the coding or noncoding regions of the same gene, and results in a compensatory change that abrogates the deleterious effect of the germline mutation (3) (**Figure 1C**). In autosomal recessive disorders caused by compound heterozygous mutations, intragenic recombination can lead to reversion through the generation of a wild-type allele with the other allele carrying both germline mutations (3) (**Figure 1D**). Copy-neutral loss of heterozygosity (CN-LOH) can eliminate the chromosomal region encompassing the germline mutation and replace it with a copy of the wild-type chromosome from the other parent (3) (**Figure 1E**). Furthermore, in autosomal dominant disorders caused by gain-of-function mutations, the elimination of a dominant germline mutation can be achieved by aneuploidy resulting from chromosomal structural mutations such as chromosomal deletion and chromothripsis (3) (**Figure 1F**).

Reversion mosaicism has been described in several disorders that affect the hematopoietic and nonhematopoietic systems such as Bloom syndrome, Fanconi anemia, dyskeratosis congenita, tyrosinemia, and epidermolysis bullosa (3, 4). Reversion mosaicism has frequently been found in diseases that affect highly generating tissues and organ systems, which may reflect the significant cell proliferation and the high mutagenicity of these tissues (5). Primary immunodeficiency diseases (PIDs) are a major category of disorders in which somatic reversion has been frequently described (4). The first case of reversion mosaicism in PIDs was recognized because of a milder than expected clinical phenotype in a patient with adenosine deaminase (ADA) deficiency (6). Reversion mosaicism has subsequently been found in some PIDs such as X-linked severe combined immunodeficiency (X-SCID) (5, 7–13), recombination activating gene 1 (RAG1) deficiency (14, 15), CD3ζ deficiency (16–19), and Wiskott–Aldrich syndrome (WAS) (20–36). The establishment of reversion mosaicism has modified the clinical phenotype of these disorders in which revertant cells have a selective advantage *in vivo* (5). The beneficial consequence of somatic reversion has paved the way to gene therapy, based on gene addition through viral-mediated transfer of the wild-type copy of the gene, which has been applied for curative therapy for ADA-SCID, X-SCID, and WAS (37, 38). Here, we review the newer findings of reversion mosaicism in PIDs and describe clinical implications for understanding this phenomenon.

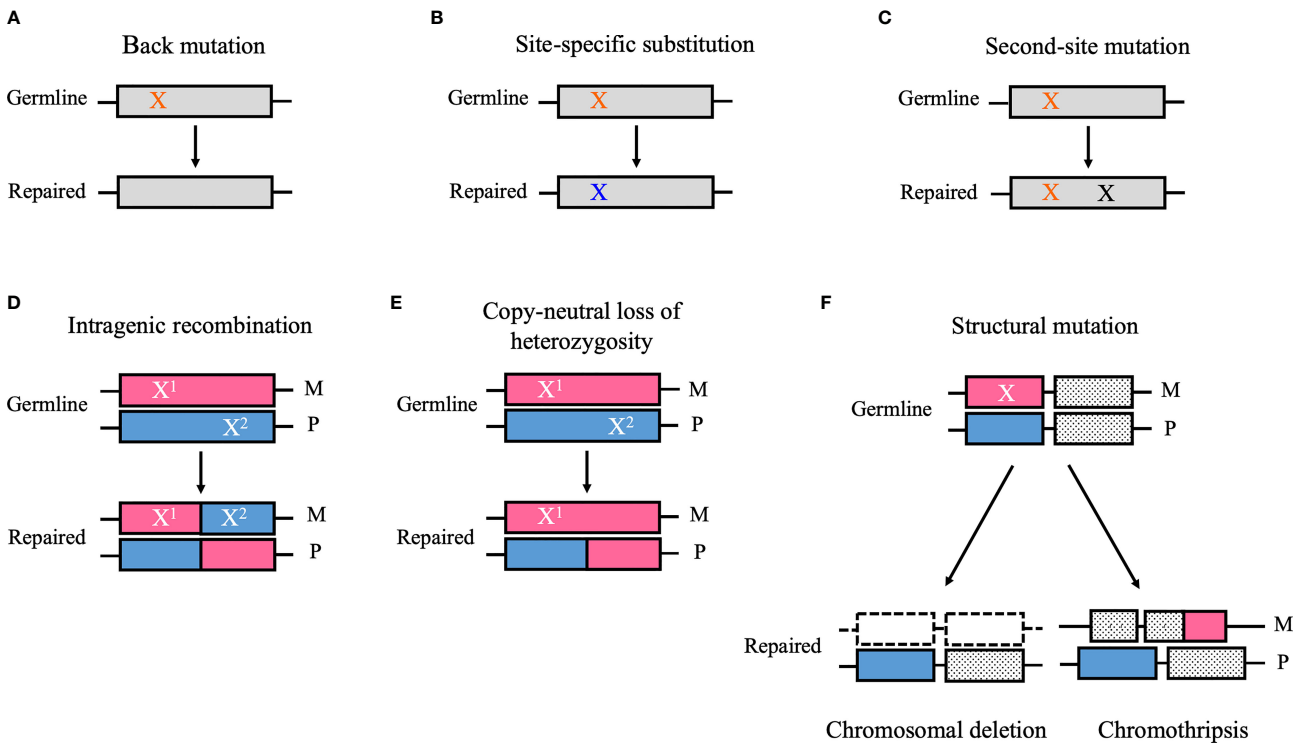

FIGURE 1 | Schematic diagrams of the reversion mechanism. **(A)** A germ line point mutation (orange "X") is changed to the wild-type sequence (i.e., back mutation). **(B)** A germ line point mutation substitutes for a nucleotide other than the wild-type sequence (blue "X"), which restores the original amino acid sequence or results in the alternation to a less deleterious amino acid than in the original germline mutation (i.e., site-specific substitution). **(C)** A mutation that occurs at a different site from the germline mutation but within the coding or noncoding regions of the same gene (black "X") can cause a compensatory change that abrogates the deleterious effect of the germline mutation (i.e., second-site mutation). **(D)** Intragenic recombination can lead to reversion through the generation of a wild-type allele, whereas the other allele carries both germline mutations (i.e., X^1 and X^2). **(E)** Copy-neutral loss of heterozygosity can eliminate the chromosomal region encompassing the germline mutation and replace it a copy of the wild-type chromosome from the other parent. **(F)** A structural mutation such as chromosomal deletion and chromothripsis can abrogate a deleterious gain-of-function mutation ("X") by modifying the chromosomal structure. M, maternal allele; P, paternal allele. **(A)** Back mutation, **(B)** Site-specific substitution, **(C)** Second-site mutation, **(D)** Intragenic recombination, **(E)** Copy-neutral loss of heterozygosity, **(F)** Structural mutation.

PIDS ASSOCIATED WITH REVERSION MOSAICISM

WAS

WAS is a rare X-linked disorder characterized by thrombocytopenia, eczema, susceptibility to infections, autoimmunity, and lymphoreticular neoplasia. The responsible gene, *WAS*, encodes the WAS protein (WASp). WASp, an essential regulator of actin cytoskeleton remodeling, is only expressed in hematopoietic cells and is involved in multiple functions in immune cells. WASp-deficient T-cells fail to form and stabilize the immunological synapse, thereby resulting in defective polarization of T-cells toward antigen-presenting cells. WASp also has a critical role in T- and B-cell interaction, B-cell homeostasis, phagocytosis, cytolytic function of natural killer (NK) cells, and FAS-mediated apoptosis. Various roles of WASp in innate and acquired immunity have been progressively reported, but the full function of WASp remains to be clarified (39–42).

Somatic reversion seems to occur frequently in WAS patients, as can be expected from the fact that at least 40 cases in 17 studies have been published to date (20–36) (**Table 1**). The European Society for Immunodeficiencies survey of 40 groups/laboratories worldwide documented that WASp reversions occurred in approximately 11% (30/272) of WAS patients (22). WASp-expressing revertant cells have been detected as early as 3 months and as late as the 4th decade of life. Revertant cells have been detected among T-cells, B-cells, and NK cells. However, T-cells are the most common subset of lymphocytes to contain revertant cells with percentages ranging from 5% to 80% (**Table 1**). The fraction of revertant $CD4^+$ and $CD8^+$ T-cells is diverse among each case. In approximately one-half of the observed cases, more than one lymphoid lineage included revertant cells; however, no cases of revertant cells involving myeloid cells have been reported (4, 22, 39) (**Table 1**). The revertant cells can accumulate over time and are preferentially represented among memory T-cells, regulatory T-cells, and NK cells, which indicates that a reversion mutation can confer significant selective advantage to the revertant cell population when it restores WASp function (27, 29, 30, 33–35). The expansion of revertant cells, which has been observed in *in vitro* culture, also supports this notion (21). An important note is that the appearance of WASp revertant cells in circulation does not necessarily confer a clinical benefit for the WAS phenotype. In many patients, the presence of revertant cells does not prevent the occurrence of life-threatening complications, whereas several patients have presented with a milder clinical course than expected, based on their mutations (40). Clinical outcome after reversion probably depends on at least following factors: the timing of reversion during cell differentiation process, the type and function of cells in which reversion occurs, and the size and diversity of revertant cell population (27). In addition, autoimmune manifestations may be caused by the presence of residual WASp-negative autoreactive lymphocyte *per se*, even if WASp-expressing revertant cells accumulate (27, 28). These findings will offer valuable perspectives on the possible clinical outcomes of gene therapy for WAS patients.

Based on a molecular point of view, back mutations and second-site mutations are equally common mechanisms that restore WASp expression (4, 39) (**Table 1**). The mutational hotspots for reversion have not been indicated; however, 30% of reversions occur in exon 10, as distinct from the fact that most loss-of-function mutations in *WAS*, primarily missense mutations, are in exons 1 to 4 (22, 43). Of note, more than 30 different genotypic changes have been detected in primary T-cell clones from an adult WAS patient carrying the c.995C>T nonsense mutation in exon 10 (24, 26). However, revertant cells with multiple genotypes within the same individual has been identified in two WAS siblings who carried a c.58C>T nonsense mutation in exon 1 and in other two siblings who carried a c.G1305del mutation, which is predicted to cause a truncated protein that lacked the C-terminal verprolin homology, cofilin homology, and acidic domains (23, 25). This phenomenon also has been identified in other immunological disorders (e.g., CD3ζ deficiency, X-SCID, and RAG1 deficiency) and nonimmunological disorders (e.g., epidermolysis bullosa) (11, 14, 19, 44). Conventional direct sequencing analysis may not

TABLE 1 | Somatic revertant cases of WAS.

Number of patients	Type of reversion	Revertant cell	Reference
1	Second-site mutation	Lymphocytes	(20)
1	Back mutation	$CD4^+$T, $CD8^+$T	(21)
3	A 6-bp deletion (DNA slippage)	$CD4^+$T, $CD8^+$T	(29, 31)
2	Second-site mutation (19-bp deletion)	$CD4^+$T, $CD8^+$T, B	(30, 32)
1	Back mutation (1-bp deletion)	NK	(33)
1	Second-site mutation	$CD4^+$T, $CD8^+$T, NK	(34)
1	Back mutation (1-bp insertion)	T, B, NK	(35)
1	Back mutation	$CD4^+$T, $CD8^+$T, γδT	(36)
30[a]	Back mutation or second-site mutation	T, B, NK	(22)
2	Multiple second-site mutations	$CD4^+$T, $CD8^+$T, B, NK	(23)
1	Multiple reversions (back mutation, site-specific substitutions and second-site mutations)	$CD4^+$T, $CD8^+$T, B	(24, 26)
2	Multiple second-site mutations	$CD4^+$T, $CD8^+$T, B	(25)
1	Second-site mutation	$CD4^+$T, $CD8^+$T	(27)
1	Back mutation	$CD4^+$T, $CD8^+$T, NK	(28)

[a]*Some cases overlap.*

WAS, Wiskott–Aldrich syndrome; NK, natural killer.

allow the detection of multiple changes if they do not represent a significant fraction of existing genotypes. Therefore, a possibility is that the frequency of multiple revertant genotypes in WAS (and possibly other genetic disorders) has been under-reported because of technical reasons. More sensitive and efficient technologies such as deep sequencing technologies and droplet digital polymerase chain reaction (PCR) can be effective for detecting minimal fraction in reversion mosaicism. However, the fact that the clinical history of WAS patients often allows long-term management in the absence of resolutive treatment may allow sufficient time for revertant cells of diverse genotypes to individually accumulate (26, 40).

The reason for the high incidence of reversion mosaicism in WAS, compared to other PIDs, is unclear. The possible mutagenic effect of chronic antibiotic exposure or the high proliferation rate in lymphoid cells and consequently increased DNA polymerase mistakes, which are derived from inadequate immune response of WAS patients to infectious agents, may have a causative role in mutagenicity in WAS patients (40). However, no known involvement of WASp in DNA replication, proofreading, and DNA repair exists, and the general mutation rate does not appear to be increased in WAS patients (26). In addition to cytoplasmic functions, several nuclear functions of WASp such as gene transcription and the maintenance of genomic stability have recently been elucidated (45–52). Recent research has demonstrated that nuclear WASp is critical in preventing the accumulation of a genome-destabilizing nucleic acid structure in human T-cells that is called the "R-loop" (i.e., a three-stranded nucleic acid structure consisting of an RNA : DNA duplex and a displaced nontemplate single strand DNA) (51). It also has an important role in correcting existing double strand breaks in human B-cells (52). Moreover, another study revealed a novel function of WASp in the DNA-damage-induced Golgi dispersal response, and its disruption as a contributor to radiosensitivity in T- and B-cells (53). WASp accordingly has critical functions in maintaining a stable genome in human T- and B-cells. The precise mechanism in the high frequency of reversion mosaicism in WAS patients remains unclear; however, WASp-deficiency-induced genomic instability may have an important role in the high incidence of somatic reversions in WAS patients.

ADA Deficiency

ADA deficiency is an autosomal recessive disorder that represents a SCID phenotype caused by the excessive accumulation of toxic purine nucleoside metabolites. Enzyme replacement therapy (ERT) with polyethylene glycol-ADA eliminates toxic metabolites and protects lymphocytes, thereby restoring the immune function. ERT is efficient for temporal adjunct therapy before hematopoietic stem cell transplantation (HSCT) or gene therapy (6, 54–58). In the middle of 1990s, Hirschhorn et al. reported two unrelated ADA-SCID patients who presented with progressive clinical improvement and biochemical and immunological remission (6, 54). They believe that the patients' clinical course could have been modified by somatic reversion in which revertant cells had a selective advantage; however, because of the absence of parental genetic analysis, a cause of the genetic mosaicism in one of the patients could not be distinguished from a postzygotic somatic mutation (6, 54).

ERT has been used because it causes a rapid improvement in immune reconstitution, even in somatic mosaicisms caused by reversion mutations. In three of four revertant patients previously reported who underwent ERT, wild-type or second-site revertant cells decreased markedly during ERT, although its biochemical and immunological effects and clinical outcomes differed among them (55–58). This finding highlights the possibility that ERT reduces the selective advantage of revertant cells. However, immune reconstitution and clinical improvement in these patients could depend on several factors such as the lineage of cells in which reversion occurred and the duration of ADA exposure. These findings provide insights into the immunological and clinical effects of gene therapy, especially in the management of ADA patients by combining gene therapy with ERT, with regard to the appropriate timing and duration of ERT.

Gene therapy is a therapeutic strategy for ADA-SCID patients and increasing evidence suggests that T memory stem cells (T_{SCM}) are expected to be a potential target because of its long-persisting memory and stem-cell nature (59). Notably, recent study with ADA-SCID patients uncovered that gene-corrected T_{SCM} can persist and preserve functional T-cell pool *in vivo* for up to 12 years without oncogenic feature (59). This finding indicates that T_{SCM} gene correction is a crucial strategy for the T-cell-based gene therapy.

X-SCID

X-SCID is the most frequent form of SCID, which is caused by mutations in the gene encoding the common gamma chain (γc) of the interleukin-2 receptor (*IL2RG*). In the absence of a functional γc, early lymphoid progenitor cells are unable to achieve the normal development of T-cells and NK cells. Most patients who present with SCID in infancy have a poor survival beyond 2 years without immune reconstitution therapy. However, hypomorphic mutations or reversion mutations of the *IL2RG* gene could result in a mild phenotype (i.e., late-onset combined immunodeficiency). In previous reports, five of seven revertant patients actually recovered γc expression and presented with the mild phenotype (5, 7–13) (**Table 2**). Some of these patients exhibited polyclonal expansion of the T-cell receptor variable β (TCR Vβ) repertoire and a restored response to mitogens (5, 11). In these cases, reversion may occur in progenitor T-cells before the stage in which T-cells undergo TCR rearrangement. In addition, in recently reported cases, reversion was detected in NK cells or B-cells and T-cells, thereby indicating that reversion may occur at the level of the T/NK progenitor or common lymphoid progenitor (12, 13). By contrast, in another report, a patient had multiple reversions with a different proportion in each cell lineage (11) (**Table 2**). This observation suggests that reversion may occur more frequently than previously believed and may have the potential to ameliorate immunological and clinical presentations of X-SCID.

By contrast, an adverse clinical effect of reversion was raised by the observation of an X-SCID patient who presented with an Omenn syndrome (OS)-like phenotype (9) (**Table 2**). In this patient, revertant T-cells were only detected in skin infiltrates, not in peripheral blood, which indicated that the clonal expansion of

TABLE 2 | Clinical and genetic features of revertant cases of X-SCID.

Germline mutation	Type of reversion	Revertant cell	Clinical impact	Reference
c.343T>C	Back mutation	$CD4^+T$, $CD8^+T$	Patient presented with a mild phenotype, but subsequentially underwent HSCT because of recurrent infections	(7)
IVS1+5G>A	Second-site mutation	T (only skin infiltrated)	Omenn syndrome	(9)
c.466T>C	Back mutation	αβT, γδT	Mild phenotype	(10)
c.284-15A>G	Multiple reversions	$CD4^+T$, $CD8^+T$	Mild phenotype	(11)
c.655T>A	Back mutation	$CD4^+T$, $CD8^+T$, γδT	Mild phenotype	(5)
c.260T>C	Back mutation	$CD4^+T$, $CD8^+T$, B	Mild phenotype	(12)
c.172C>A	Back mutation	$CD8^+T$, NK	Patient died of graft failure and fungal infection after HSCT	(13)

X-SCID, X-linked severe combined immunodeficiency; HSCT, hematopoietic stem cell transplantation; NK, natural killer.

revertant cells in response to local factors such as infections or autoantigens may be implicated in such a distinctive phenotype. This case indicates that somatic reversion is a possible cause of clinical improvement and for diverse and complicated presentations.

RAG1 Deficiency

RAG1 is a component of the RAG complex promoting V(D)J recombination in precursor lymphocytes by which highly diverse immunoglobulins and TCR genes are generated. Severe/null defects in the *RAG1* gene cause T^-B^-SCID, whereas leaky mutations and somatic reversions are responsible for the development of OS (14, 15, 60, 61). In a previous report of a patient with RAG1 deficiency who presented with the OS phenotype, multiple second-site mutations that generated a partially functional RAG1 molecule could have contributed to insufficient immunological reconstitution (14). In another report, a true back mutation, which was believed to occur in a limited pool of progenitor T-cells, may have been involved in the presentation of the OS phenotype in a patient (15). Both of these patients had a restricted pattern in the TCR Vβ repertoire and activated T-cell markers. In the first patient, not all mutations were commonly shared by $CD4^+$ and $CD8^+$ T-cells (14). However, a complete defect in RAG activity results in differentiation arrest before the stage of double-negative to double-positive transition (62). These findings suggest that other precedent mutations may be followed by these second-site mutations, which occur after CD4/CD8 lineage commitment.

CD3ζ Deficiency

CD3ζ, also called as CD247, is a subunit of the TCR complex that is required for its assembly and for surface expression, which is important for TCR-mediated signal transduction. TCR complexes lacking the CD3ζ chain cumulate in the Golgi apparatus instead of moving onto the plasma membrane and are shunted to lysosomes for degradation. The CD3ζ chain contributes to peripheral T-cell activation and intrathymic T-cell differentiation. The lack of CD3ζ expression results in a severe but incomplete block of T-cell differentiation at the double-positive stage (16, 17, 63).

Three patients with CD3ζ deficiency were previously found to have somatic reversion mutations in *CD3Z* gene (16–19). The first patient, who carried a germline mutation within the intracellular first immunoreceptor tyrosine-based activation motif domain and presented with the SCID phenotype, harbored three second-site mutations that partially rescued membranous TCR expression but functioned poorly (16). Ten percent of T-cells were revertant T-cells expressing normal levels of the TCR-CD3 complex and were polyclonal. All of the cells were $CD4^+$ T-cells carrying one of three reversion mutations. Each of the three mutations was found in populations harboring different rearrangements of TCR Vβ genes. This finding suggests that the reversions may occur before the V(D)J recombination at the double-negative stage. The lack of revertant cells in $CD8^+$ T-cells indicates that these three mutations may probably have little selective advantage for $CD8^+$ T-cells. The second revertant case involved a germline mutation within the initiation codon, which inhibited translation. This patient harbored revertant cells with a true back mutation or a compensatory second-site mutation, which caused restoration or substitution of the initiation codon; however, the frequency of revertant cells in T-cell compartment was very low (17). Revertant T-cells are capable of expanding in response to TCR stimuli *in vitro* but may not be sufficient to repopulate the T-cell compartment and achieve immunological reconstitution *in vivo*. The *CD3Z* gene may have intrinsic mutability so as to result in multiple reversions, as reported in two patients (18). This finding has been substantiated by the latest report of a revertant patient with multiple second-site mutations who presented with the mild phenotype (19). This patient carried a homozygous 2-bp deletion mutation (c.43_44delCA) within *N*-terminal signal peptide in the *CD3Z* gene. Deep sequencing analysis revealed 52 somatic variants of which 49 variants restored the reading frame, most of which retained the characteristic amino acid distribution functioning as signal peptides but were not fixed to the primary sequence. A surprising finding is that 1 year after the first analysis, 23 somatic variants, which included nine novel variants, were detected with a different proportion from that of the first analysis, which may reflect the varying antigen stimulations. The multiple variation in the amino acid sequence caused by reversion mutations may reflect that signal peptides permit the fluctuation of the amino acid sequence, which may be distinct from the integral sequence for protein function. This case demonstrates that the location of

the germline mutation can affect the spectrum of the revertant pool.

Other PIDs Presenting With the SCID Phenotype

The first case of revertant mosaicism in Janus kinase 3 (JAK3) deficiency was reported in a consanguineous family with two affected siblings (64). They presented with a relatively mild phenotype with CD4 lymphopenia, which manifested as combined immunodeficiency. A novel homozygous missense mutation (c.3196T>C) was identified in both patients. One of these patients presented with somatic mosaicism by a back mutation in $CD8^+$ T-cells; in the other patient, the same back mutation was detected in $CD4^+$ and $CD8^+$ T-cells. The patient with $CD4^+$ and $CD8^+$ revertant T-cells presented with a milder phenotype than her counterpart. Therefore, somatic reversion in $CD4^+$ T-cells may have contributed to the attenuation of disease severity. However, JAK3-signaling analysis showed that the presence of revertant cells had no effect on the residual JAK3-dependent signaling. Hence, the hypomorphic nature of this mutation rather than reversion mutation may have been associated with the milder phenotype in the second patient.

In a study of Chinese patients with DNA ligase IV (LIG4) deficiency, one of seven patients presented with reversion mosaicism (65). The germline genotype of this patient was a compound heterozygote (c.833G>T; c.935delC) in the *LIG4* gene; however, wild-type clones and simultaneously generated clones, which contained both inherited mutations, were detected with TA cloning analysis. This finding indicates that intragenic recombination was a probable mechanism of somatic repair of *LIG4* mutations. The reversion event may occur at an early stage of embryonic development because these clones were obtained from T-cells, NK cells, granulocytes, and oral mucosa cells in different proportions. However, the somatic reversion was insufficient to reconstitute clinical and immunological phenotype in this patient.

A patient with interleukin (IL)-7 receptor α deficiency, who had compound heterozygous mutations (one single nucleotide variant and one intragenic copy number variant involving one exon), presented with an atypical clinical course and late onset (66). Mosaicism for the wild-type allele in the single nucleotide variant position was unexpectedly identified in whole exome sequencing. However, whether it originated from somatic reversion, maternal engraftment, or both was unproven.

XL-EDA-ID (NEMO Deficiency)

X-linked anhidrotic ectodermal dysplasia with immunodeficiency (XL-EDA-ID) is caused by hypomorphic mutations in *IKBKG* gene, which encodes nuclear factor-κB (NF-κB) essential modulator (NEMO). NEMO has an important role in activating inhibitor of NF-κB (IκB) kinase, which phosphorylates and degrades IκB to activate NF-κB. A defect in NEMO causes various abnormalities in the signal transduction pathway involving NF-κB, including the IL-1 family protein receptors, the Toll-like receptors, CD40, and the tumor necrosis factor (TNF) receptor (67, 68). Patients with XL-EDA-ID present with various immunological phenotypes such as reduced production of proinflammatory cytokines in response to lipopolysaccharide and IL-1 family protein stimulation, dysregulated immunoglobulin synthesis, defective anti-polysaccharide antibody synthesis, and NK cell dysfunction (67, 68).

In a Japanese nationwide survey, as many as 90% (9/10) of XL-EDA-ID patients presented with somatic reversion mosaicism (69). In these patients, most revertant cells were detected in $CD4^+$ and/or $CD8^+$ T-cells, a low number of revertant cells was detected in B-cells, and no revertant cells were detected in monocytes. One sibling who harbored a duplication mutation in the *IKBKG* gene also showed reversion mutation in approximately 50% of $CD56^+$ NK cells (70). In two siblings, the revertant $CD8^+$ T-cells primarily presented with the memory/effector phenotype and the restricted pattern of the TCR Vβ repertoire (69, 70). The high incidence of somatic mosaicism may reflect a strong selective advantage for NEMO-expressing revertant cells *in vivo*, especially in the T-cell lineage. However, the precise role for NEMO in the development and homeostasis of each cell lineage has not been fully elucidated. The clinical impacts of somatic mosaicism in XL-EDA-ID patients were not demonstrated in the studies (69, 70).

By contrast, the clinical impact of NEMO reversion has been demonstrated in another revertant patient who presented with refractory inflammatory colitis (71). The mechanism underlying the NEMO colitis was demonstrated in a mouse model of intestinal epithelium-specific NEMO deficiency in which intestinal epithelial cells exhibited increased sensitivity to TNFα-induced apoptosis and caused disruption of the epithelial barrier, resulting in chronic intestinal inflammation (72). In this patient, the NEMO-deficient intestinal epithelium might be further damaged by TNFα-producing mononuclear cells because of the reversion mutation of NEMO. The patient's peripheral TNFα-producing cells reduced with repeated anti-TNFα antibody administrations, thereby causing the clinical improvement. Thus, NEMO reversion may have a deleterious role in the intestinal inflammation.

Owing to the presence of a pseudogene (i.e., *IKBKGP1*), the genetic diagnosis of XL-EDA-ID is difficult to determine when using only genomic DNA sequencing analysis. It should be confirmed by sequencing analysis of NEMO cDNA or long-range PCR amplicon using primers that do not amplify the pseudogene. The presence of somatic mosaicism can cause a misdiagnosis of XL-EDA-ID when a normal revertant cDNA sequence can be selectively amplified with PCR or when most of each cell lineage consists of NEMO-expressing revertant cells (69).

LAD-1

Leukocyte adhesion deficiency type 1 (LAD-1) is caused by a genetic defect in the *ITGB2* gene, which encodes the common chain of the β2 integrin family (CD18). The adhesion of leukocytes to the endothelium is primarily defected, thereby causing abnormal leukocyte extravasation. Patients are usually affected with recurrent bacterial infections and impaired wound healing without pus formation. The severity of the clinical phenotype is directly associated with the degree of CD18 deficiency (73). To date, five revertant patients harboring

CD18-expressing cells have been reported in LAD-1 (74–76). The first patient had a compound heterozygous mutation and carried a reversion to the normal sequence in one of the disease-causing mutations only within a small fraction of $CD8^+$ T-cells. This fraction was monoclonal, which indicated that reversion may occur in a committed hematopoietic lineage and eventually gain a selective advantage (74). In three additional patients, all of whom presented with gastrointestinal manifestations, 5% to 20% of revertant cells were detected among $CD8^+$ T-cells and exhibited a restricted pattern of the TCR Vβ repertoire (75). In all four cases, revertant $CD8^+$ T-cells represented the memory/effector phenotype. In addition, a functional study revealed that reversion mutations showed the recovery of superantigen-induced proliferation and adhesion to specific ligands *in vitro* (75). Taken together, these findings suggest that reversion mutations may exhibit functional recovery and a proliferative advantage and lead to the acquisition of the immunological memory in response to antigen stimulation, especially in $CD8^+$ T-cells.

In these previous patients, susceptibility to infections was not ameliorated, probably because of the lack of reversion in granulocytes. By contrast, as with the three revertant patients with gastrointestinal manifestations, the latest case of LAD-1 in a patient, who was presumably a revertant patient because of the presence of minimal $CD18^+$ fraction in circulating $CD8^+$ T-cells, was also affected with severe colitis, which was endoscopically compatible with Crohn's disease (76). The inflammatory response at barrier sites such as the oral mucosa and probably in the skin and gastrointestinal tract in LAD-1 may be caused by infections because of relative tissue neutropenia and by a defect in the phagocytosis of apoptotic neutrophils by tissue macrophages (i.e., efferocytosis), which acts as signal to down-regulate IL-23 and IL-17 responses (77). However, the clinical impact of the presence of CD18-expressing CD8+ T-cells regarding the gastrointestinal inflammatory response remains to be clarified.

XLP-1

X-linked lymphoproliferative disease type 1 (XLP-1) is caused by loss-of-function mutations in *SH2D1A*, which encodes SLAM-associated protein (SAP). Patients with XLP-1 are highly susceptible to Epstein–Barr virus (EBV) infection because of the impaired activation and cytotoxicity of $CD8^+$ T-cells. They also develop hypogammaglobulinemia because of impaired $CD4^+$ T-cell function (78, 79). In the first study of somatic reversion in XLP-1, eight patients represented a small fraction of SAP^+ revertant cells in $CD8^+$ T-cells, except for one patient who also presented with SAP^+ fraction in NK cells (78). Most revertant $CD8^+$ T-cells lay in the $CD45RA^-CCR7^-$ effector memory T-cell compartment and maintained a stable number over decades. In addition, SAP^+ revertant $CD8^+$ T-cells showed more proliferation than did the SAP-deficient counterpart and exhibited functional recovery in response to EBV-specific stimulus. As an alternative, in the latest Japanese nationwide survey of XLP-1, three of 18 patients, who were alive at the time of this study, had a longer survival without HSCT, despite having a history of EBV infection (79). A remarkable finding is that all three patients had 3% to 7% of the SAP^+ fraction in $CD8^+$ T-cells, and one patient had a smaller fraction of SAP^+ cells in $CD4^+$ T-cells. SAP^+ $CD8^+$ T-cells mostly lay in the effector memory T-cell population and showed proliferation and functional recovery in response to EBV-specific stimulus, compatible to those of healthy controls. In addition, in the patient who harbored revertant SAP^+ cells in the $CD4^+$ T-cell population, intracellular IL-10 expression and inducible costimulatory expression were predominantly observed in the revertant SAP^+ $CD4^+$ T-cells. This finding suggested that the reversion event conferred partial reconstitution of humoral immunity in this patient. The findings in these studies collectively suggested that a reversion mutation provides the ability for proliferation and the acquisition of effector function for revertant $CD8^+$ T-cells in response to B-cells (i.e., the reservoir of EBV) and eventually leads to expansion of revertant T-cells in correlation with EBV infection. The fact that a relatively small fraction of revertant cells modified the fatal clinical phenotype of XLP-1 suggested that gene therapy may potentially be an effective strategy for XLP-1.

DOCK8 Deficiency

Dedicator of cytokinesis 8 (DOCK8) is a guanin nucleotide exchange factor for the Rho-GTPase CDC42, turning it into the active, GTP-bound form. GTPase activation induces dynamic actin cytoskeleton rearrangement, leading to immunological synapse formation, migration, adhesion, and cytolytic granule release. DOCK8 also exhibits an actin-independent function, including the regulation of STAT3 phosphorylation and nuclear translocation. Hence, DOCK8 deficiency impacts innate and adaptive immune responses (80).

Of note, somatic reversions occur in 13% to 50% of patients with DOCK8 deficiency (81, 82). The high frequency of somatic reversions may reflect the *DOCK8* gene's location within a recombination hotspot that is characterized by many subtelomeric repetitive sequences (81). Reversion is detected more frequently in T-cells, especially in a high proportion of $CD8^+$ T-cells, rather than in NK or B-cells, but it is not detected in monocytes (81, 82). Revertant T- and B-cells exhibit expansion *in vitro* and *in vivo* and restore $CD8^+$ T-cell cytotoxicity, $CD4^+$ T-cell cytokine production, and memory B-cell generation (82). These findings indicate that DOCK8 somewhat exerts a proliferative and survival advantage in numerous lymphoid lineages and has a key role in many fundamental aspects of lymphocyte biology. DOCK8-expressing cells are predominantly enriched in the memory compartment of $CD4^+$ T-cells and B-cells rather than in the corresponding naïve compartment. By contrast, the difference in the proportions of DOCK8-expressing cells in the $CD8^+$ T-cell population was not as significant as in $CD4^+$ T-cells and B-cells (81, 82). This finding reflects a possible differential role of DOCK8 among each lymphoid lineage and in $CD4^+$ and $CD8^+$ T-cell differentiation. Based on a clinical perspective, the increase in revertant cells can delay the progression of the disease, but do not necessarily reconstitute the whole clinical phenotype and do not abrogate the need for HSCT (81, 83–85). By contrast, the three patients reported by Pillay *et al.* exhibited restored protein expression and spontaneous improvement in the clinical

phenotype (82). These findings from these patients provide evidence that gene therapy is a promising prospect for treating DOCK8 deficiency.

CARD11 Deficiency

In 2015, the first case of somatic reversion in caspase recruitment domain-containing protein 11 (CARD11) deficiency was reported (86). The patient was one of two Turkish siblings who were born to consanguineous parents. She had postnatally acquired cytomegalovirus infection and presented with the OS-like phenotype, which included erythroderma and lymphadenopathy. She carried a homozygous germline nonsense mutation (c.450C>A) in the coiled-coil domain in the *CARD11* gene, which impaired NF-kB signaling and IL-2 production. A somatic second-site mutation (c.449G>T) was detected in the same codon, which restored protein expression, thereby leading to a partial functional restoration in a subset of T-cells. The revertant cells were mostly in tissue-infiltrating $CD4^+$ and $CD8^+$ T-cells, but not in granulocytes and fibroblasts, with a highly restricted T-cell repertoire. This finding indicated that reversion may occur before CD4/CD8 lineage commitment in progenitor T-cells. As demonstrated in mouse studies, CARD11 is also essential for the thymic development of $FoxP3^+$ regulatory T-cells, which significantly contributes to peripheral tolerance of T-cells (87, 88). These siblings indeed had a lack of regulatory T-cells in the peripheral blood or in lymph node biopsy. Along with the selective advantage of virus-specific revertant T-cells in response to the persistent stimuli by chronic cytomegalovirus infection, the lack of regulatory T-cells may also have a causative role in the development of the OS-like features.

ARPC1B Deficiency

Actin-related protein 2/3 complex subunit 1B (ARPC1B) is a component of the actin-related protein 2/3 complex, which interacts with WASp to induce actin polymerization and generate new branched actin filament networks in the context of cell migration, endocytosis, vesicular trafficking, and cytokinesis (89). ARPC1B exerts a regulatory role for the assembly and maintenance of the actin-related protein 2/3 complex, and its disruption results in morphological, functional, numerical aberrations in platelets, defect in neutrophil motility and chemotaxis, and functional deficiency in T-cells and NK cells (89–93). In 2017, somatic reversion in ARPC1B deficiency was first reported in two patients from unrelated families (89). In these patients, somatic reversion restored ARPC1B protein expression and was associated with a restricted TCR repertoire. One patient harbored revertant cells within the $CD8^+$ T-cell compartment, but not in $CD4^+$ T-cells and B-cells. The other patient harbored revertant cells in the $CD8^+$ T-cell and NK cell compartments. In addition, $ARPC1B^+$ revertant $CD8^+$ T-cells displayed an improvement in T-cell migration. These findings suggested that reversion mutations in *ARPC1B* provide a preferential advantage in $CD8^+$ cytotoxic T-cells and NK cells, which is consistent with the fact that the absence of ARPC1B disrupts the proliferation capacity of cytotoxic T-cells (93). Furthermore, revertant $CD8^+$ T-cells were only enriched in effector memory T-cells, T effector memory-RA^+ cells, and T_{SCM}, but not in naïve and central memory T-cells. This finding indicated that part of the selective pressure may have occurred on antigen stimulations, thereby leading to the acquisition of the immunological memory. Neither patient in the study seemed to have an improvement in their clinical phenotype and one of the patients underwent HSCT due to recurrent infections and refractory autoimmune/autoinflammatory manifestations. The restoration of ARPC1B expression may partially reconstitute T-cell function and proliferation; however, more observations are required to assess whether genetic reconstitution provides the clinical improvement in ARPC1B deficiency.

MYSM1 Deficiency

Myb-like, SWIRM, and MPN domains 1 (MYSM1) is a histone deubiquitinase that specifically deubiquitinates the K119-monoubiquitinated form of histone 2A, a chromatin marker of gene transcription silencing. MYSM1 reverses the transcription repression of genes that are involved in hematopoietic stem cell homeostasis, hematopoiesis, and lymphocyte differentiation (94). Spontaneous *in vivo* reversion to normal was recently reported in a male patient with MYSM1 deficiency who harbored a missense mutation (c.1967A>G) that affected the stability of the protein and damaged histone deubiquitinase activity (94). He presented with a complete lack of B-cells, T-cell lymphopenia, bone marrow failure, and developmental abnormalities. During the course of his treatment, he showed spontaneous improvement in the number of lymphoid and myeloid lineages. Analysis of bone marrow mononuclear cells revealed the complete restoration of B-cell development. Circulating B-cells exhibited polyclonal pattern of B-cell receptor IgM and IgG repertoire. A surprising finding was that a genetic reversion to normal was detected in virtually all hematopoietic stem cells (HSCs), and subsequently detected in approximately 100% of circulating B-cells, NK cells, and monocytes, and in 63.5% of circulating T-cells. These findings suggested that the reversion event may have provided a selective advantage for these cells *in vivo* and completely corrected immunological abnormalities and bone marrow failure in this patient. This observation suggests that clinical application of gene therapy will be effective for immunodeficiency and bone marrow failure in patients with MYSM1 deficiency.

WHIM Syndrome

Warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome is usually caused by a gain-of-function mutation in the *CXCR4* gene, which is in chromosome 2. CXCR4 signaling is a suppressive modulator of HSC and neutrophil migration from bone marrow. Its gain-of-function mutation results in myelokathexis, which is a characteristic of this disorder. In addition, the *CXCR4* gain-of-function mutation may cause a multisystem and combined immunodeficiency disease because of its broad expression in hematopoietic and nonhematopoietic cell types (95). A female patient with WHIM syndrome, designated as WHIM-09, experienced a spontaneous phenotypical remission (96). In this patient, chromothripsis, a complex genetic process characterized by scattering, rearrangement, inversion and deletion of genomic element on one or a few chromosomes (97), affected one copy of chromosome 2 and deleted 164 genes, including the mutated copy of *CXCR4*, probably in a single HSC from the

patient. This event conferred to the modified HSC a strong selective advantage over germline mutated cells and corrected the defects in the myeloid and erythroid lineage. By contrast, the lymphoid lineage and epithelial cells were not affected by the reversion event. Therefore, the patient had a somatic mosaic of WHIM cells and reverted non-WHIM cells. The patient has been healthy for at least 20 years since the estimated time point of the reversion event. These findings suggest that the adaptation of genome editing technology to inactivate the mutant *CXCR4* allele in autologous HSC is a potential curative strategy for WHIM syndrome.

GATA2 Deficiency

GATA-binding protein-2 (GATA2) is a transcription factor that is involved in the development of HSCs and is essential for definitive hematopoiesis. Heterozygous mutations in *GATA2* results in GATA2 haploinsufficiency and causes qualitative impairment in HSC function. GATA2 deficiency is characterized by various hematopoietic and nonhematopoietic features such as multilineage cytopenias (especially monocytopenia and B-cell and NK cell lymphopenia), hematologic malignancies, immunodeficiency, pulmonary alveolar proteinosis, lymphedema, and sensorineural hearing loss (98, 99). The first somatic revertant case of GATA2 deficiency was revealed by the diagnosis of a man's two affected sons who presented with the typical phenotype (98). They harbored a heterozygous mutation in *GATA2* (c.216C>A; p.Y72*), which caused a premature stop codon and loss of the two DNA-binding zinc fingers and the nuclear localized signal. By contrast, their father was asymptomatic and displayed normal immunophenotyping. A surprising finding was that he harbored the pathogenic mutation, which was identical with that of his sons, in sperm and skin fibroblasts. However, 93% of leukocytes carried the silent somatic mutation (c.216C>T; p.Y72Y), which indicated somatic reversion. Sorted monocytes, T-cells, B-cells, and NK cells carried this silent somatic variant in heterozygosity, which indicated that somatic reversion may occur at the HSC stage. The patient remained asymptomatic with no hematopoietic or nonhematopoietic manifestations at the point of time in this study when he was 61 years old, despite the fact that he did not carry the silent somatic variant in the nonhematopoietic lineage. This finding indicates that the restoration of hematopoietic cells is sufficient for preventing the occurrence of hematopoietic and nonhematopoietic manifestations in GATA2 deficiency. These observations suggest the potential advantage of gene therapy. However, more observations are necessary for understanding the full details of somatic reversion in GATA2 deficiency.

SAMD9/SAMD9L Syndrome

Germline mutations in sterile alpha motif domain protein 9 (*SAMD9*) and its paralogue SAMD9-like (*SAMD9L*), which are located in tandem on chromosome 7q21, are associated with human syndrome with a propensity for bone marrow failure and myelodysplastic syndrome (MDS) with monosomy 7 and 7q deletion (100, 101). *SAMD9* and *SAMD9L* gain-of-function mutations represent a strong growth-suppressive effect because of the defective endosomal turnover of cytokine receptors such as the epidermal growth factor receptor (100). Somatic reversion events, which remove the mutant *SAMD9* and *SAMD9L* allele, occur *via* monosomy 7, chromosome arm 7q deletion, second-site loss-of-function mutation, and CN-LOH (102–110). Cells that lose the mutant allele may gain a proliferative advantage, relative to the growth restriction imposed in mutation-carrying cells, and thereby result in reversion mosaicism. Any somatic reversion event may be temporarily beneficial for hematopoiesis; however, the loss of the germline mutated copy *via* monosomy 7 and 7q deletion results in haploinsufficiency (3, 100, 108). In fact, in an original case series of myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, enteropathy syndrome, which was initially recognized as a disease caused by *SAMD9* gain-of-function mutations, two of 11 patients developed myelodysplastic syndrome (MDS) with monosomy 7 (102). Several additional cases have subsequently been reported and affected patients have a propensity to develop MDS/acute myeloid leukemia with monosomy 7 and 7q deletion (102, 106, 107, 109). Like patients with *SAMD9* mutations, patients with *SAMD9L* gain-of-function mutations are also predisposed to the development of MDS/acute myeloid leukemia with monosomy 7 and 7q deletion (108, 109). CN-LOH and second-site loss-of-function mutation can apparently rescue the hematopoietic defect without MDS/acute myeloid leukemia predisposition in both disorders (100, 103–105, 107–110).

In the recent investigation of pediatric MDS cohort, 8% of the consecutively diagnosed patients harbored germline *SAMD9*/*SAMD9L* mutations (101). Of the patients with *SAMD9*/*SAMD9L* mutations, 61% underwent somatic reversion, of whom 51% had benign (CN-LOH or second-site mutation) and 95% had maladaptive nature (monosomy 7 and 7q deletion). Furthermore, bone marrow single-cell sequencing revealed multiple competing reversions in individual patients. As shown in the study, somatic reversion in SAMD9/SAMD9L syndrome is highly prevalent and diverse, and changes in clonal diversity may modify clinical outcomes of the patients.

DISCUSSION

Over the past decades, observations of somatic reversion in PIDs have provided significant insights into the function of genes in different cell types, the frequency and mechanism of genetic changes, and clinical consequences because of the presence of revertant cells. These observations have yielded the notion that reversion events provide a wide-spectrum of molecular and clinical effects in PID patients. In fact, the molecular mechanisms that restore gene expression and function vary from single nucleotide substitutions to large deletions or insertions. Moreover, chromosomal structural change, as seen in WHIM syndrome and SAMD9/SAMD9L syndrome, contributes to complete gene abrogation in PIDs caused by gain-of-function mutations (96, 102–110).

The increased number of reversions in PIDs suggests that reversion mutations may occur much more frequently than previously believed rather than being a significantly rare event that occurs in distinctive situations. The relatively high occurrence of reversion is substantiated by the fact that

independent reversions can occur in respective siblings (30, 64, 79) and in respective cell lineages (81). In addition, reversion mutations have been also discovered in myeloid and erythroid lineages and in nonhematopoietic cells, whereas lymphoid lineages are most frequently affected (65, 94, 96, 98, 105, 106) (**Table 3**). Together with these observations, reversion mutations can occur frequently in various cell types and differentiation stages and can be detected when affected cells acquire a proliferative advantage, which can be determined by, at least, the following factors: the type of cell, the timing of reversion, the lifespan of revertant cells, the nature of mutant genes, and extrinsic factors such as persistent antigen stimulations (3, 5, 78). Based on this perspective, even when various cell lineages share the same reversion, identical reversions may occur independently at more differentiated stages rather than from a single less-differentiated common progenitor.

Reversion mutations frequently occur in some PIDs, although susceptible genes and locations may not be random. For example, distinctive sequences in *WAS* have been implicated in reversion mutation by means of DNA slippage mechanism or hairpin loop formation (29–32). In addition, the homologous sequence of *IKBKG* (i.e., pseudogene) and subtelomeric repetitive sequences in the *DOCK8* locus may have contributed to the recombination-mediated somatic repair (70, 81, 82). (**Table 3**). Loss-of-function mutations in genes that are involved in genomic stability such as *BLM*, *LIG4*, *FANCA*, *FANCC*, *FANCD2*, and presumably *WAS* may be associated with a high incidence of reversion mutations (3, 51–53). Gene variation analysis, used to evaluate the mutation frequency of PID genes in which reversion events have or have not been described, has revealed that genetic variation is significantly greater for revertant genes than for nonrevertant or control genes, especially in coding sequences (18). The study also demonstrated that the presence of CpG islands was more frequent in revertant PID genes than in nonrevertant genes; however, other factors such as local chromatin structure and accessibility for DNA repair may influence mutation frequency (18). These intrinsic gene properties in collaborating with long-term management using mutagenic agents and prompt cell turnover due to chronic infections and inflammation (which may induce DNA polymerase mistakes) may be involved in the high incidence of reversion mutations.

As observed in previous reversion cases in PIDs, reversion mutations are predominantly found in T-cell populations (**Tables 1**-**3**). This fact may reflect the consequence of the selective occurrence of reversion mutations in T-cell lineage. Extensive intrathymic cell division and the existence of self-renewing, long-persisting memory T-cells—that is, T_{SCM}—may account for the selective reversion detection in T-cells (3, 89, 111). As an alternative, reversion mutations may occur in various cell lineages with the same probability or in less-differentiated hematopoietic progenitor cells. In this scenario, selective

TABLE 3 | Other PIDs in which somatic reversion has been detected.

Disease	Type of reversion	Revertant cell	Reference
ADA deficiency	Back mutation Second-site mutation	$CD4^+$T, $CD8^+$T, B, NK	(6, 54–58)
RAG1 deficiency	Back mutation Second-site mutation	$CD4^+$T, $CD8^+$T	(14, 15)
CD3ζ deficiency	Back mutation Second-site mutation	$CD4^+$T, $CD8^+$T, NK	(16–19)
XL-EDA-ID	Loss of the duplicated region Back mutation	$CD4^+$T, $CD8^+$T, B, NK	(69–71)
LAD-1	Back mutation Site-specific substitution Second-site mutation	$CD8^+$T, NK	(74–76)
XLP-1	Back mutation Site-specific substitution	$CD4^+$T, $CD8^+$T, NK	(78, 79)
DOCK8 deficiency	Back mutation Second-site mutation CN-LOH Intragenic recombination Loss of the duplication/deletion mutation	$CD4^+$T, $CD8^+$T, B, NK	(81–85)
JAK3 deficiency	Back mutation	$CD4^+$T, $CD8^+$T	(64)
DNA ligase IV deficiency	Intragenic recombination	T, NK, granulocytes, oral mucosa	(65)
CARD11 deficiency	Second-site mutation	$CD4^+$T, $CD8^+$T	(86)
ARPC1B deficiency	Back mutation	$CD8^+$T, NK	(89)
MYSM1 deficiency	Back mutation	T, B, NK, monocytes	(94)
WHIM syndrome	Chromothripsis	myeloid and erythroid lineage	(96)
GATA2 deficiency	Site-specific substitution	T, B, NK, monocytes	(98)
SAMD9/SAMD9L syndrome	Monosomy 7 Deletion of 7q Second-site mutation CN-LOH	BM and PB cells (including myeloid and lymphoid lineage)	(102–110)

PID, primary immunodeficiency disease; NK, natural killer; CN-LOH, copy-neutral loss of heterozygosity; BM, bone marrow; PB, peripheral blood.

proliferation of revertant T-cells may be caused by any or all of the following factors: homeostatic expansion on the background of T-cell lymphopenia, intrinsic gene function for T-cell proliferation, and persistent antigen-specific responses (3) (**Figures 2A, B**). In the context of the basic pathology with bone marrow failure or neutropenia—that is, the function of an affected gene being equally essential for some or all hematopoietic lineages—reversion mutations have been detected in cells other than lymphoid lineages, as observed in SAMD9/SAMD9L syndrome, MYSM1 deficiency, WHIM syndrome, GATA2 deficiency, and Fanconi anemia (94, 96, 98, 105, 106, 112) (**Table 3** and **Figure 2C**). For the aforementioned reasons, reversion mutations may be predominant in T-cell populations.

The detection of somatic mosaicism by using conventional standard Sanger sequencing is challenging because the variant peak can be misinterpreted as background noise on the chromatogram when revertant cells constitute a minimal fraction (2). Highly sensitive and efficient molecular technologies such as deep sequencing technologies and quantitative techniques such as droplet digital PCR allow the detection and quantification of low-frequency mosaicism (1–3, 19, 113). These technologies can permit the efficient and accurate sequential observation of mosaicism, which will reveal important matters such as the relationship between spontaneous mosaicism oscillation and the clinical phenotype, the effect of alternative treatment on mosaicism [e.g., ADA deficiency (55–58) and XL-EDA-ID (71)], and the therapeutic effect of gene therapy (1). Furthermore, emerging sensitive and innovative methodologies, especially "single-cell" approaches, will facilitate reversion detection and the evaluation of characteristics, behavior, and fate of a revertant cell (3, 113).

Owing to overlapping and complicated phenotypes, the diagnosis of PIDs is not necessarily easy. Reversion mutations can modify clinical and immunological phenotypes; therefore, diagnostic delay and underdiagnosis can occur, particularly when the disease intrinsically presents with diverse clinical and immunological features, as seen in XL-EDA-ID (69).

Furthermore, the technical limitations and the nature of the gene may make a genetic diagnosis difficult. For example, transcriptomics is often used as a complementary diagnostic tool if patients remain without a molecular diagnosis after target next-generation sequencing, whole exome sequencing, and whole genome sequencing analysis; however, a molecular diagnosis can be delayed, if revertant wild-type allele is selectively amplified (84). In addition, if most blood cells are revertant cells, a molecular diagnosis can be hampered by the major detection of revertant wild-type alleles. Therefore, genetic

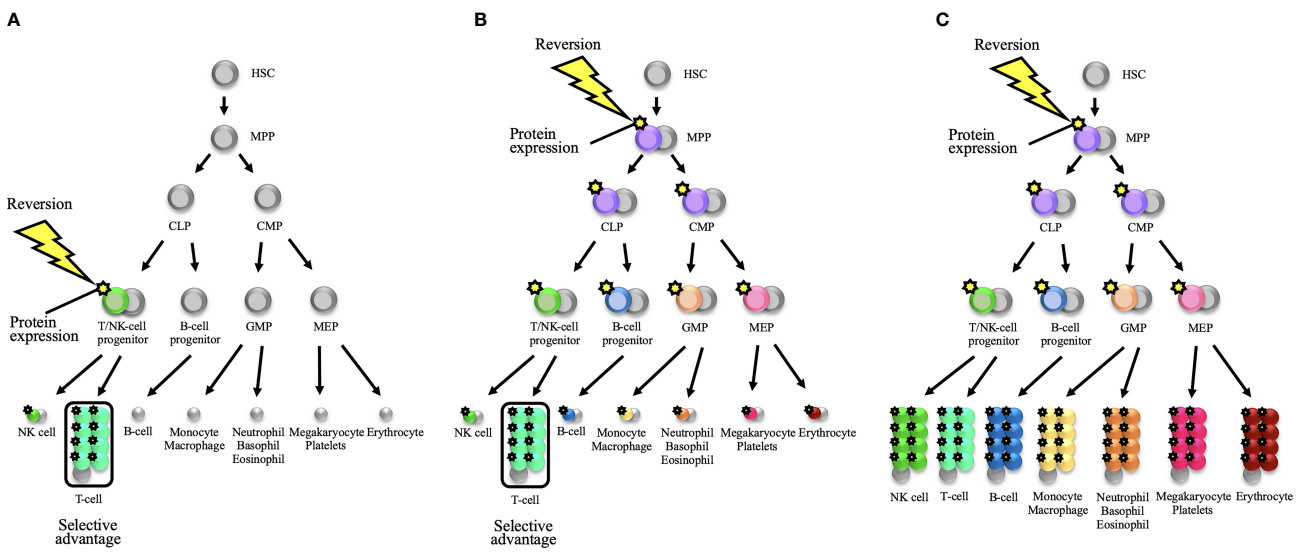

FIGURE 2 | Schematic representations of somatic reversion in the hematopoietic system that eventually results in distinct hematological and immunological reconstitution. **(A)** A somatic reversion event can occur at a late stage of the hematopoietic hierarchy and provide a selective advantage to a restricted lineage. The diagram shows the occurrence of reversion in a T/NK-cell progenitor. When T-cell lineage selectively exhibits a proliferative advantage, somatic mosaicism will be detected only in the T-cell population. This model is applicable to the reversion mosaicism in the context of severe combined immunodeficiency or a T-cell deficiency. **(B)** A somatic reversion event can occur in less-differentiated hematopoietic progenitor cells. For example, when reversion mutation occurs in a multipotent progenitor (MPP), cells from all hematopoietic lineages can theoretically harbor the same reversion mutation. However, when the T-cell lineage selectively represents a proliferative advantage, somatic mosaicism will seemingly be detected only in the T-cell lineage. This selective advantage may depend on intrinsic gene function for T-cell proliferation and/or persistent antigen-specific responses. In disorders with T-cell lymphopenia such as severe combined immunodeficiency, homeostatic expansion will further provide a positive effect on proliferative advantage over the T-cell lineage. **(C)** When the germline mutation is deleterious for all hematopoietic lineages (i.e., the function of the affected gene is equally essential for all hematopoietic lineages), the reversion mutation in less-differentiated progenitor cells can reconstitute the whole hematopoietic system. In this scenario, somatic mosaicism can be detected in all compartments. Bone marrow failure disorders may be applicable to this model. HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; MEP, megakaryocyte-erythroid progenitor; NK, natural killer.

analysis on DNA extracted from a tissue other than peripheral blood such as buccal mucosa cells and, optimally, skin fibroblasts may be advisable, particularly when a concern exists about a PID for which the reversion occurrence is relatively high such as WAS, DOCK8 deficiency, and XL-EDA-ID (3).

In summary, the clinical impact of somatic reversion depends on the type, differentiation, diversity, and number and function of cells in which reversion occurs, intrinsic function of an affected gene, and location of the germline mutation. In the context of the adaptation of gene therapy for PID patients, cells harboring artificial genomic changes should work as expected *in vivo*. To date, the mechanism of reversion and the function of revertant cells have been elucidated by means of emerging technologies, even in genes and in cell types in which reversion events have not been described. In the future, further technological evolutions will enable scientists to generate new perspectives for reversion mosaicism in PIDs.

AUTHOR CONTRIBUTIONS

HM and TW wrote and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Endocrinopathies in Inborn Errors of Immunity

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Inborn errors of immunity (IEI), caused by hereditary or genetic defects, are a group of more than 400 disorders, in which the immune system, including lymphocytes, neutrophils, macrophages, and complements, does not function properly. The endocrine system is frequently affected by IEI as an associated clinical feature and a complex network of glands which regulate many important body functions, including growth, reproduction, homeostasis, and energy regulation. Most endocrine disorders associated with IEI are hypofunction which would be treated with supplementation therapy, and early diagnosis and appropriate management are essential for favorable long-term outcomes in patients with IEI. In this review, we aimed to comprehensively summarize and discuss the current understanding on the clinical features and the pathophysiology of endocrine disorders in IEI. This review is composed with three parts. First, we discuss the two major pathophysiology of endocrinopathy in IEI, autoimmune response and direct effects of the responsible genes. Next, the details of each endocrinopathy, such as growth failure, hypothyroidism, hypoparathyroidism, adrenal insufficiency, diabetes mellitus (DM) are specified. We also illustrated potential endocrinopathy due to hematopoietic stem cell transplantation, including hypogonadism and adrenal insufficiency due to glucocorticoid therapy.

Keywords: endocrinopathy, thyroiditis, diabetes mellitus, growth failure, inborn errors of immunity (IEIs), HCT

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INTRODUCTION

Inborn errors of immunity (IEI), caused by hereditary or genetic defects, are a group of more than 400 disorders, in which the immune system, including lymphocytes, neutrophils, macrophages, and complements, does not function properly (1–4). While the clinical characteristics of each IEI differs, defects in the functions of the immune system generally lead to increased susceptibility to infection, which can be life-threatening or can cause permanent damage to various organs. Although the clinical symptoms of IEI are generally present at birth or in early childhood, patients can be affected with IEI at any age (1–4).

In addition to opportunistic infections, various clinical features are also involved in IEI. Autoimmunity and abnormal inflammation in the absence of apparent infection have often been observed clinically in association with IEI, and IEI has been linked to specific autoimmune/allergic

complications at various frequencies (5). Furthermore, numerous causative genes of IEI are involved in the fundamental functions of cell differentiation and proliferation and are shared by various types of cells. Taken together, the clinical problems caused by the impaired molecular function of IEI genes are not limited to immunological systems (5).

Clinical complications of IEI can be classified into three groups according to the causes, the direct organ damages from opportunistic infections, chronic autoimmune/allergic inflammation, and impaired molecular functions of the causative genes (2, 5). Further, abnormal cell proliferation or carcinogenesis due to IEI could be form the pathogenesis of endocrinopathy. In any case, every organ can be affected by IEI, and for the clinical management of IEI, interventions for both opportunistic infections and clinical complications are essential.

The endocrine system is not an exception and is frequently affected by IEI as an associated clinical feature (**Table 1**) (12). The endocrine system is a complex network of glands that produce and release hormones which regulate many important body functions, including growth, reproduction, homeostasis, and energy regulation. During childhood, the endocrine system is essential for acquiring normal growth and secondary sexual characteristics (13), and pediatric endocrinopathy seriously affects long-term outcomes. On the other hand, most endocrine disorders associated with IEI are hypofunctions that can be treated with supplementation therapy. Therefore, early diagnosis and appropriate management are essential for favorable long-term outcomes in patients with IEI. In this review, we aim to summarize and discuss the current understanding of the clinical features and pathophysiology of endocrine disorders in IEI.

PATHOPHYSIOLOGY OF ENDOCRINOPATHY IN IEI

Autoimmune

The immune system becomes self-tolerant through mechanisms called "tolerance". Induction of tolerance is accomplished by education of both B and T cells, which occurs in both central (bone marrow and thymus) and peripheral (spleen and lymph nodes) lymphoid organs, and immune dysregulation causes autoimmune/allergic responses (14–17). Autoimmune diseases can be classified into systemic diseases, such as systemic lupus erythematosus, rheumatoid arthritis (RA), and systemic sclerosis, and organ-specific diseases, such as type 1 diabetes mellitus (T1DM) and autoimmune thyroiditis (17). Endocrine organs are the major targets of organ-specific autoimmune diseases, and most endocrinopathies in IEI are presumably mediated by autoimmunity (14–16).

The most common autoimmune endocrine disorders are autoimmune thyroid disorders and T1DM (2). Despite extremely rare conditions, hypophysitis, adrenalitis, ovarian failure, and hypoparathyroidism can occur due to an autoimmune response. Autoimmune endocrine disorders are characterized by associations with autoantibodies and/or autoreactive lymphocytes, which result from an interaction between environmental factors and genetic predisposition. For a genetic contribution to autoimmune endocrinopathies, epidemic studies of twins provide robust evidence that monozygotic twins have a higher concordance rate for disease than dizygotic twins, and familial accumulation is frequently observed. Most autoimmune endocrine disorders have been reported to be associated with specific major histocompatibility complex (MHC)/human leukocyte antigen (HLA) molecules.

Despite the long history of intensive investigations, the precise genetic mechanisms underlying autoimmune endocrine disorders are poorly understood. One of the possible explanations is the "autoimmune surveillance of hypersecreting mutants" (ASHM) hypothesis. The presence of autoreactive T cells whose nature is detecting self-antigens from hormone secretion pathway, dedicates to detect and to remove malfunctioned endocrine cells which potentially disrupt organismal homeostasis. The speculation looks persuasive, nevertheless, the speculation does not clarify the reason why certain endocrine organs, such as thyroid gland and beta cells, tend to be more affected.

A recent significant expansion of our understanding of monogenic IEI, which is associated with autoimmune endocrine disorders, would provide valuable insights into their pathophysiology.

Classically, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and immunodeficiency, polyendocrinopathy, and enteropathy X-Linked syndrome (IPEX) exemplify monogenic autoimmune disorders associated with endocrine disorders (**Figure 1**). From the discovery of the *AIRE* gene in APECED (18, 19), defects in central tolerance with alteration of self-antigen expression levels in the thymus would be a cause of T1DM, and strictly controlled negative selection of T cells through tissue-specific self-antigen expression in the thymus is essential for preventing autoimmune endocrine disorders (20). Studies of another IEI associated with T1DM, IPEX syndrome have shown that the responsible gene, *FOXP3* (21, 22), plays a critical role in the function of $CD4^+CD25^+$ regulatory T (Treg) cells (23, 24), and its defects cause loss of peripheral Tregs, leading to autoreactive T cell activation and proliferation (20, 23, 24).

Recent studies have revealed that other molecules are involved in monogenic autoimmune disorders involving endocrinopathies. In cohorts of subjects with an IPEX-like phenotype, up to half do not exhibit pathogenic variant in *FOXP3*, and recent investigations revealed that a number of gene defects involved in Treg cell function cause an IPEX-like phenotype, such as IL2RA, STAT5b, GOF in STAT1/3, and LRBA (**Figure 1**) (25–32).

Additionally, several monogenic disorders in immune function have also been reported to be involved in T1DM. Immune checkpoint proteins play a central role in balancing effective immune responses to pathogens, as well as regulating autoimmune responses against self-tissues. CTLA4 is one of the components, and heterozygous deficiency of the protein gives rise to inappropriate polyclonal T-cell activation (10, 33, 34),

TABLE 1 | Modified the classification of the International Union of Immunological Societies Expert Committee according to complications of endocrinopathy (2).

Category		Disease	Gene	Inheritance	OMIM	Endocrinopathies						
						growth failure	thyroiditis	diabetes mellitus	adrenal insufficiency	hypoparathytoidism	hypogonadism	dyslipidemia
Immunodeficiencies affecting cellular and humoral immunity	T-B- SCID	Artemis deficiency	DCLRE1C	AR	605988	X						
		Ligase 4 deficiency	LIG4	AR	601837	X						
	Combined immunodeficiency (CID), generally less profound than SCID	Polymerase and deficiency	POLD1, POLD2	AR	174761, 600815	X						
Combined immunodeficiencies with associated or syndromic features	DNA repair defects	Ataxia-telangiectasia	ATM	AR	607585			X			X	*
		Bloom syndrome	BLM	AR	604610	X		X				
		Ligase I deficiency	LIG1	AR	126391	X						
		MCM4 deficienc	MCM4	AR	602638	X			X			
		POLE1 (Polymerase ϵ subunit 1) deficiency (FILS syndrome)	POLE1	AR	174762	X						
		POLE2 (Polymerase ϵ subunit 2) deficiency	POLE2	AR	602670		X	X				
		RNF168 deficiency (Radiosensitivity, Immune Deficiency, Dysmorphic features, Learning difficulties [RIDDLE] syndrome)	RNF168	AR	612688	X						
	Thymic defects with additional congenital anomalies	Chromosome 11q deletion syndrome (Jacobsen syndrome)	11q23del	AD	147791	X						
		Chromosome 10p13-p14 deletion syndrome (10p13-p14DS)	Del10p13-p14	AD	601362	X				X		
		DiGeorge/velocardio-facial syndrome Chromosome 22q11.2 deletion syndrome (22q11.2DS)	Large deletion (3 Mb) typically in chromo- some 22 (TBX1)	AD	602054					X		
		CHARGE syndrome	CHD7	AD	214800	X					X	**
	Immuno-osseous dysplasias	Immunoskeletal dysplasia with neurodevelopmental abnormalities (EXTL3 deficiency)	EXTL3	AR	617425	X						
		MYSM1 deficiency	MYSM1	AR	612176	X						
		MOPD1 deficiency (Roifman syndrome)	RNU4ATAC	AR	601428	X						
		Schimke immuno-osseous dysplasia	SMARCAL1	AR	606622	X						
	Hyper IgE syndromes (HIES)	PGM3 deficiency	PGM3	AR	172100	X						
	Other defects	KMT2A deficiency (Wiedemann-Steiner syndrome)	KMT2A	AD	605130	X						
		Kabuki syndrome (type 1 and 2)	KMT2D KDM6A	AD XL	602113 300128	X						
		Activating de novo mutations in nuclear factor, erythroid 2- like (NFE2L2)	NFE2L2	AD	617744	X						
		STAT5b deficiency	STAT5B	AR	245590	X						
		STAT5b deficiency	STAT5B dominant negative	AD	604260	X						
Predominantly antibody deficiencies	Severe reduction in all serum immunoglobulin isotypes with profoundly decreased or absent B cells, agammaglobulinemia	X-linked agammaglobulinemia (XLA)	BTK	XL	300300	X						
		NFKB1 deficiency	NFKB1	AD	164011		X					
		NFKB2 deficiency	NFKB2	AD	615577				X			***
		Activated p110δ syndrome (APDS) type2	PIK3R1	AD	616005			X				†
Diseases of immune dysregulation	Regulatory T cell defects	IPEX, immune dysregulation, polyendocrinopathy, enteropathy X-linked	FOXP3	XL	300292		X	X				
		CTLA4 Haploinsufficiency with autoimmune infiltation	CTLA4	AD	616100		X	X	X			††
		STAT3 GOF mutation	STAT3	AD	102582		X	X				
		LRBA deficiency	LRBA	AR	614700		X	X				
	Autoimmunity with or without lymphoproliferation	APECED (APS-1), autoimmune polyendocrinopathy with	AIRE	AR or AD	240300	X		X	X	X	X	

(Continued)

TABLE 1 | Continued

Category		Disease	Gene	Inheritance	OMIM	Endocrinopathies							
						growth failure	thyroiditis	diabetes mellitus	adrenal insufficiency	hypoparathytoidism	hypogonadism	dyslipidemia	
		candidiasis and ectodermal dystrophy											
		ITCH deficiency	ITCH	AR	606409		X	X					
		JAK1 GOF	JAK1	AD GOF	147795	X	X						
Congenital defects of phagocyte number or function	Congenital neutropenias	Glycogen storage disease type 1b	G6PT1	AR	602671							X	
		Kostmann Disease, SCN3	HAX1	AR	605998						X		†††
		JAGN1 deficiency	JAGN1	AR	616012								
		P14/LAMTOR2 deficiency	LAMTOR2	AR	610389	X							
		Barth Syndrome (3-Methylglutaconic aciduria type II)	TAZ	XL	300394	X							
	Defects of motility	β actin deficiency	ACTB	AD	102630	X							
		Leukocyte adhesion deficiency type 2 (LAD2)	SLC35C1	AR	605881	X							
Defects in intrinsic and innate immunity	Predisposition to mucocutaneous candidiasis	mucocutaneous candidiasis	STAT1 GOF	AD GOF	600555		X	X					
	Type 1 interferonopathies	Congenital Osteopetrosis	TNFSF11	AR	602642	X							
Autoinflammatory disorders	Type 1 interferonopathies	Spondyloenchondro-dysplasia with immune dysregulation (SPENCD)	ACP5	AR	171640	X							
Bone marrow failure		Bone marrow failure	DKCB5	AR	615190	X							
		Fanconi Anemia, TypeA, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P, Q, R, S, T, U, V, W	FANCA FANCB FANCC BRCA2 FANCD2 FANCE FANCF XRCC9 FANCI BRIP1 FANCL FANCM PALB2 RAD51C SLX4 ERCC4 RAD51 BRCA1 UBE2T XRCC2 MAD2L2 RFWD3	AR or XLR	227650 300514 227645 605724 227646 600901 603467 614082 609053 609054 614083 618096 610832 613390 613951 615272 617244 617883 616435 617247 617243 617784								
		MIRAGE (myelodysplasia, infection, restriction of growth, adrenal hypoplasia, genital phenotypes, enteropathy)	SAMD9	AD GOF	617053				X		X		
		Coats plus syndrome	STN1, CTC1	AR, AR	613129, 617053								

References of additional information are indicated as follows.

** (6): ** (7): *** (8).*

† (9): †† (10): ††† (11).

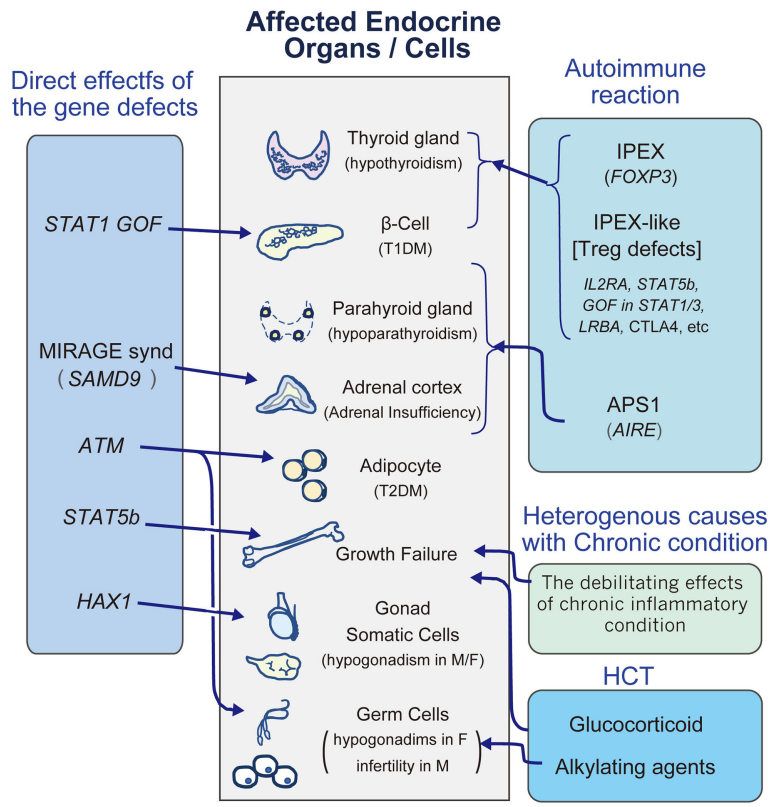

FIGURE 1 | A diagram to review the major endocrinopathies in IEI. M, male; F, female; HCT, hematopoietic stem cell transplantation; T1DM, type1 diabetes mellitus; T2DM, type2 diabetes mellitus.

leading to highly variable features of autoimmune responses, which may include endocrinopathies of adrenal insufficiency, T1DM, and thyroiditis (35). On the other hand, anti-CTLA-4 antibody therapy, which is a novel therapeutic approach to malignancy by targeting immune checkpoint proteins, is associated with serious immune-related adverse events, such as autoimmune hypophysitis (36, 37).

Endocrinopathy would be comorbidity of recently identified IEI. Inherited PD-1 deficiency and activated PI3Kδ syndrome (APDS) type 2, would associate with T1DM (38–40). Familial thyroiditis and T1DM are reported in patients with A20 (TNFAIP3) haploinsufficiency (41, 42).

Autoimmune endocrine disorders are progressive and are considered untreatable and irreversible. Generally, the treatment of endocrinopathy involves hormone supplementation instead of immunological therapy. However, the experience of endocrine disorders in IEI would provide valuable insights for developing novel therapeutic approaches for autoimmune endocrine disorders. Early hematopoietic cell transplantation (HCT) for IPEX appears to reverse the autoimmune complications, including T1DM, suggesting that Treg cells may be more tractable for the induction of tolerance and could be a possible target for the radical treatment of T1DM (9, 17, 43). Consistently, administration of ruxolitinib, an inhibitor of Janus kinase (JAK) 1 and 2 reversed hyperglycemia of T1DM in a case of STAT1 GOF (44).

Direct Effects of the Responsible Genes

Some endocrine disorders in IEI are thought to be caused by the direct influence of molecular defects (**Figure 1**).

STAT5b is a transcription factor that activates the transcription of *IL-2Rα*, *FOXP3*, and *Bcl-2*, and its deficiency exhibits combined immunodeficiencies with a reduced number of Treg cells and increased IgE (27, 45). The JAK2 and STAT pathways (STAT1, STAT3, STAT5A, and STAT5B) are also involved in the growth hormone (GH) receptor pathway, and we will discuss the details in C-2.

Kostmann disease is a type of severe congenital neutropenia (SCN) caused by *HAX1* deficiency (46). HAX-1 is involved in the regulation of apoptosis and plays a role in myeloid differentiation. Cekic et al. investigated seven female patients with severe neutropenia due to the p.Trp44X variant in the *HAX1* gene, and all female patients exhibited ovarian insufficiency. *HAX1* mRNA is abundantly expressed in the testes and ovaries, and the authors speculated that the *HAX1* gene plays an important role in ovarian development (11).

MIRAGE syndrome is a rare condition characterized by myelodysplasia, recurrent infection, restriction of growth,

adrenal hypoplasia, genital phenotypes, and enteropathy. It is an autosomal dominant disorder typically caused by a *de novo* pathogenic variant of *SAMD9/SAMD9L*, whose gain of function variant increases the role of the molecule as a negative regulator of cellular proliferation by altering the endosome system (47, 48). This results in cytopenia, bone marrow failure, and immunodeficiency. In patients with MIRAGE syndrome, the adrenal glands are hypoplastic with severely disorganized structures, resulting in adrenal insufficiency. These findings are distinct from those of Addison's disease caused by autoimmune inflammation (47–50).

ATM (ataxia telangiectasia, mutated) is essential for DNA repair, and its defects lead to ataxia telangiectasia (AT) with multisystem syndrome, including combined immunodeficiencies (51). Diabetes mellitus (DM) with severe insulin resistance is a major complication of AT. A study of AT mice model revealed that ATM regulates adipocyte differentiation, and in AT patients, adipocyte differentiation is disturbed, resembling lipodystrophy, which is one of the major causes of insulin resistance (52). *ATM* is also essential for germ cell meiosis. In mice, *Atm* deficiency results in severe meiotic disruption as early as leptonema of prophase I (53), resulting in germ cell depletion. Consistently, hypogonadism in AT is common only in female patients (6), because in contrast to testes, ovarian development is germ cell dependent.

STAT1 gain-of-function (GOF) variants impair the development of IL-17-producing T cells, resulting in mucocutaneous candidiasis (28, 31). It also causes autoimmune endocrinopathies, such as thyroiditis and T1DM. In a meta-analysis, autoimmune thyroiditis and T1DM were observed in 23.0% and 5% of *STAT1* GOF patients, respectively (54). On the other hand, recent *in vitro* and *in vivo* studies have demonstrated an essential role for STAT1 in islet cell death. *Stat1* ablation in T1DM model mice (NOD mice) ameliorated diabetes and insulitis, suggesting that in addition to autoimmune mechanisms, GOF of *STAT1* would directly contribute to the pathogenesis of diabetes by inducing apoptosis of the islet beta cells (55).

CAHRGE syndrome is a genetic syndrome with known pattern of features, coloboma, heart anomaly, choanal atresia, intellectual disability, genital and ear anomalies (56, 57). Heterozygous *CHD7* gene deficiency causes most cases, and CHD7 protein regulates gene expression by chromatin remodeling (58). Immunodeficiency in CHARGE syndrome is rare, and occurs largely due to impairment in thymic development, and the severity of the immunodeficiency relates to the degree of thymic maldevelopment (7). The severest cases could be complete/near complete absence of T-cells and abnormal B-cell function with hypo-gammaglobulinemia. The syndrome is associated with impaired development of the pharyngeal arch structures, and in this regard, the clinical phenotypes overlap with 22q11.2 deletion syndrome (7). In addition to growth retardation, gonadotropin deficiency is one of the major clinical features of CHARGE syndrome. It causes micro penis in male and absent of pubertal development in both sexes (59).

ENDOCRINE DISORDERS ASSOCIATED WITH IEI

Multiorgan Endocrinopathy

APECED (APS-1)

APECED, also called autoimmune polyglandular syndrome type 1 (APS-1), is a rare condition with an approximate prevalence of 1:100,000 in US (60). and recessively inherited disorder caused by variants of the autoimmune regulator gene, *AIRE*. In addition to chronic mucocutaneous candidiasis (CMC) which is caused by the reduction of key cytokines for CMC, IL-22 and IL-17F (61), APECED is characterized by variable combinations of endocrine autoimmune diseases (62). *AIRE* consists of 14 exons spanning 11.9 kb of genomic DNA and encodes a 545-amino acid protein with a molecular weight of 58 kDa. *AIRE* plays an essential role in central tolerance induction by controlling the expression of tissue-specific self-antigens within the thymus. Disruption of *AIRE* function results in spontaneous autoimmunity in multiple organs with autoantibodies against organ specific antigens, such as TPO, Tg (thyroid gland), calcium sensing receptor (parathyroid gland), steroidogenic enzyme (adrenal cortex and gonad) and GAD/IA2/insulin (islet cells) (60).

In a classic large cohort study of 68 patients, the most common endocrinopathies were hypoparathyroidism and adrenal insufficiency (Addison's disease), both of which were observed in more than 70% of patients (63). The onset of the two conditions occurs from early childhood and can be obvious at any age. Ovary is also would be a major target of autoimmune response, and approximately 40–70% of women with APECED will develop premature ovarian failure (63–65). On the other hand, T1DM and hypothyroidism affects less than 5-10% and 20-25% of the patients, respectively (65), and the age of onset is during adulthood than childhood.

In addition to endocrinopathy, all patients exhibit oral candidiasis and other problems, such as alopecia, vitiligo, and keratopathy, which affect the patients at various frequencies, and one of the possible causes is related to autoimmunity to interleukin (IL)-17 cytokines (66).

IPEX/IPEX Like Syndrome

IPEX syndrome is caused by loss of function of X linked gene, *FOXP3* and characterized by immunodysregulation, polyendocrinopathy, enteropathy. The pathophysiology of IPEX syndrome is explained by defects for the development, survival, and/or function of Treg cells. In addition to "classic IPEX", a group of patients with an IPEX- like phenotype without pathological variant in *FOXP3* gene emerged, and several genes, such as *IL2RA, STAT5b, GOF* in *STAT1/3, LRBA* and *CTLA4*, have been identified as causative genes for IPEX-like phenotype. Recently, for categorizing the group of IPEX and IPEX like diseases, in which Treg deficiency caused by monogenic deficiency, "Tregopathies" is proposed (67).

Among the IPEX and IPEX- like patients, most common endocrinopathies are T1DM and thyroiditis. Additionally, few patients, less than 5%, develop adrenal insufficiency. In a cohort of 173 IPEX/IPEX like patients, endocrinopathy were seen at a

similar frequency (IPEX 65% vs. IPEX-like 59%). On the other hand, there was almost a two-fold difference in the frequency of patients with T1DM in the IPEX cohort compared to the IPEX-like group (IPEX 49% vs. IPEX-like 28%) (32). Clinical thyroid disease was more prevalent in the IPEX-like group (IPEX 26% vs. IPEX-like 39%), and approximately more than 70% of cases with thyroiditis are associated with T1DM (68). Of IPEX like subjects, *STAT5b* and *STAT1/3* GOF more likely evolve endocrinopathy with more than 90% of frequency (32).

Growth Failure

Endocrinologically, GH and thyroid hormone mainly contribute to linear growth, and deficiencies in these hormones lead to growth failure. However, other causes affect linear growth, e.g., undernutrition, rheumatologic inflammation, chronic renal failure, malignancy, respiratory failure, severe cardiac diseases, and genetic disease with primary effects on growth. Indeed, despite the risk of growth failure in IEI (69), GH deficiency or hypothyroidism is not documented in most IEI patients with growth failure (70). This suggests that the causes of growth failure in patients with IEI are heterogeneous.

Furthermore, glucocorticoids are strong growth suppressors, and the treatments for IEI would also cause growth failure. Few studies have evaluated treatments that affect linear growth in IEI. A retrospective study suggested that after HCT for IEI, linear growth was generally decreased rather than increased, and the glucocorticoid treatment duration for chronic graft-versus-host disease (cGVHD) was an independent risk factor for growth inhibition (71).

Among the causative genes for IEIome genes are directly involved in linear growth. The JAK-STAT signaling pathway plays an essential role in the immune system, and homozygous disruption of one of the components, STAT5b, leads to hyperimmunoglobulin E (IgE) syndrome (HIES) with low NK-cell numbers and modest T cell lymphopenia. STAT signals are also located downstream of the GH receptor (72), and a homozygous variant in *STAT5b* causes growth failure with GH insensitivity, i.e., normal serum GH levels with very low IGF-1 levels (45). Short stature in STAT5b defects is severe [-9.9 to -4.3SD), and GH therapy will not ameliorate growth failure. Although there are few data, IGF-1 therapy would be an option for short stature homozygous or heterozygous variants in *STAT5b* (73).

Although STAT3 is also involved in the GH signaling pathway, normal growth is maintained in the heterozygous loss of function of *STAT3* in HIES because STAT3 may negatively regulate GH-induced STAT5b (74, 75). Indeed, the GOF variant of STAT3 inhibits linear growth in several hematologic autoimmune disorders (75).

Thyroiditis

The thyroid gland is a butterfly-shaped organ located at the base of the neck. It releases the thyroid hormones, T3 and T4, and its serum levels are strictly regulated by the TRH–TSH–thyroid axis without apparent circadian rhythm. Thyroid hormones regulate numerous body functions by controlling metabolism. During infancy and childhood, thyroid hormones also play essential roles in growth and brain development. Therefore, diagnosis and interventions for thyroid hypofunction (hypothyroidism) during childhood should not be delayed (76).

Except for congenital hypothyroidism and iodine deficiency, most cases of hypothyroidism are caused by autoimmune thyroiditis. Autoimmune thyroiditis is one of the most common endocrinopathies, with an estimated prevalence of 350 cases/100,000/year (95% confidence interval [CI]: 280–450) in women and 60 cases/100,000/year (95% CI: 30–120) in men (77). Most patients with thyroiditis exhibit no other immunological problems, such as systemic autoimmune diseases or immunodeficiencies (78). In contrast, patients with any problems in the immune system are at a high risk for thyroiditis. IEI is not an exception, and to date, a substantial number of IEI are reported to be associated with thyroiditis (17).

The clinical symptoms and signs of hypothyroidism are insidious and non-specific. Inactivity, general fatigue, and emotional symptoms linked to depression are major symptoms of hypothyroidism in adults (78). Except for growth retardation and developmental delay, clinical symptoms are reversible by LT4 supplementation therapy, and patients at high risk for hypothyroidism should be closely monitored. Regular examination of thyroid function (TSH, fT4, and fT3) is the most efficient approach (78). Frequent evaluation of neurological development and tracking of growth with a growth chart are essential for monitoring high-risk children. Autoantibodies for thyroid glands, such as TPO-Ab and Tg-Ab, would be excellent biomarkers for autoimmune thyroiditis, and euthyroid patients with TPO-Ab and/or Tg-Ab should be carefully followed up with regularly repeated thyroid function because of the risks for evolving hypothyroidism (77, 79).

Autoimmune thyroiditis occasionally cause hyperthyroidism, namely Graves' disease. In this condition, autoantibodies that bind to the thyrotropin receptor (TSHR-Ab) stimulate thyroid gland in a TSH independent manner, leading to overproduction of thyroid hormone (80). The backgrounds of autoimmunity between hypothyroidism and hyperthyroidism are identical, and the patients with autoimmune thyroiditis are at high risk for developing hyperthyroidism. Many studies simply noted "thyroiditis", which may evolve into either hyperthyroidism or hypothyroidism, and the epidemiological details of Graves' diseases in IEI are not precisely clarified. The diagnosis of Graves' disease can be confirmed by undetectable levels of TSH (< 0.3 mU/l), high serum free T4 and T3 levels, and the presence of TSH receptor antibodies (TRAb) (80). To date, associations with selective IgA deficiency, IPEX and Wiskott–Aldrich syndrome are reported (81–86). The clinical symptoms of hyperthyroidism are insidious as hypothyroidism, and regular examination of thyroid function for high risk group are essential.

Hypoparathyroidism

The parathyroid glands produce PTH and maintain serum calcium levels. The major function of PTH is to increase bone absorption, inhibit calcium excretion from the kidney, and activate 1αhydroxylase, which synthesizes active vitamin D3, 1-25$(OH)_2$ Vit D3 (87). The serum calcium level is strictly regulated by PTH because of its essential role in numerous

biological functions. When PTH secretion is insufficient as in hypoparathyroidism, hypocalcemia develops, which can cause various clinical problems, including tetany, arrhythmia, prolonged QT interval, hypotension, and neurological complications, such as seizures (87). Aside from postsurgical hypoparathyroidism, the major causes of hypoparathyroidism include abnormal parathyroid development and autoimmune-mediated destruction (87).

22q11.2 deletion syndrome (also known as DiGeorge syndrome) is the most common microdeletion syndrome in humans, with an incidence of 1:4000 live births. Its clinical manifestations are highly variable, including congenital heart disease, palatal abnormalities, immune deficiency due to thymic hypoplasia with fewer T cells, characteristic facial features, hearing impairment, and hypoparathyroidism. The cause of hypoparathyroidism in 22q11.2 deletion syndrome is congenital defects, and the onset of hypocalcemia occurs mainly during the neonatal period. However, recent analysis revealed that autoimmune responses are involved in the pathogenesis of hypoparathyroidism, and hypocalcemia may occur at any age (88). These findings suggest that routine screening of serum calcium levels is essential for the long-term follow-up of the syndrome.

Chromosome 10p13-p14 deletion syndrome (10p13-p14DS) was originally discovered in patients with clinical phenotypes resembling 22q11.2 deletion syndrome. The syndrome also has a wide variety of clinical symptoms, including immunodeficiency and hypoparathyroidism (89, 90). Although the etiology of hypoparathyroidism in 10p13-p14 deletion syndrome is thought to be congenital defects, the involvement of autoimmune responses can be considered as 22q11.2 deletion syndrome. Further accumulation of cases with long-term follow-up of hypoparathyroidism would provide valuable insights for hypoparathyroidism mediated by autoimmune mechanisms in 10p13-p14 deletion syndrome.

The clinical features of APECED including hypoparathyroidism are discussed in C-1.

Adrenal Insufficiency

Adrenal insufficiency is a disorder that occurs when the adrenal glands are not capable of producing sufficient adrenal hormones, mainly cortisol. Cortisol protects the body from physical stress and is essential for life. Therefore, adrenal insufficiency can be life-threatening, requiring prompt and appropriate diagnosis. However, the diagnosis of adrenal insufficiency is challenging. First, the common symptoms are non-specific, such as fatigue, muscle weakness, loss of appetite, weight loss, and abdominal pain. Second, one-spot blood sampling is usually insufficient for the endocrinological diagnosis, and precise studies, including ACTH stimulation test or circadian rhythm, are required. Thus, identifying patients who are at a high risk for adrenal insufficiency is crucial (91).

The production of cortisol is strictly regulated by the hypothalamus–pituitary–adrenal (HPA) axis. Except for iatrogenic adrenal insufficiency due to glucocorticoid therapy, major causes of adrenal insufficiency are deficiency of ACTH secretion from the pituitary gland (secondary adrenal insufficiency) and organ failure of the adrenal gland (primary adrenal insufficiency) (91, 92).

Most causes of ACTH deficiency are anatomical defects of the pituitary gland, in which the production of multiple pituitary hormones is impaired. Thus, isolated ACTH deficiency is rare (93). Recently, loss-of-function variants of *NFKB2* have been shown to cause adrenal insufficiency due to isolated ACTH deficiency (8, 94, 95).

NFKB2 belongs to the NF-kB family, which is a group of evolutionarily conserved transcription factors involved in organ development, immune systems, and oncogenesis. Impaired function of NF-kB transcription factors or their regulators has been reported to be associated with primary immunodeficiency and autoimmunity (96). Classically, the condition characterized by a combination of common variable immunodeficiency (CVID) and ACTH insufficiency is called DAVID syndrome. ACTH deficiency occurred in 44% of pathologic variant carriers with a median age at onset of 8.2 years (SD ± 4.2, median 7.0, range 4–15 years) and is difficult to predict based on genetic alterations. The age at onset of ACTH deficiency suggests that the condition is acquired rather than congenital, and ACTH deficiency is presumed to be caused by T cell-mediated autoimmune diseases (8).

Except for congenital conditions, primary adrenal insufficiency is mainly caused by an autoimmune response. Isolated adrenal insufficiency accounts for up to 40% of patients with autoimmune primary adrenal insufficiency, and one or more organ-specific autoimmune endocrinopathies, such as autoimmune thyroid disease, T1DM, and premature ovarian insufficiency, can be associated with adrenal insufficiency. 21-Hydroxylase is a major autoantigen in autoimmune primary adrenal insufficiency (97), and the etiologies of primary adrenal insufficiency can be screened for using 21-hydroxylase antibodies (98). Of numerous genes that cause autoimmune adrenal insufficiency, *AIRE* is a representative gene that is associated with IEI (92). A recent cohort study of 691 patients with autoimmune adrenal insufficiency reported that variants of the *CTLA4* gene would increase susceptibility to adrenal insufficiency (99).

Recently another IEI gene has been identified to cause primary adrenal insufficiency. MCM4 (mini chromosome maintenance deficient 4 homolog) is part of a hetero-hexameric helicase complex which is important for DNA replication and genome integrity, and pathological variations were identified in an Irish travelling community manifesting with familial adrenal insufficiency (100, 101). Adrenal insufficiency is progressive and onset was usually in childhood following a period of normal adrenal function. ACTH resistance without mineralocorticoid deficiency is the characteristic of the condition. Although NK cell function is disrupted in the syndrome, the precise mechanisms of adrenal insufficiency are remained to be clarified (102).

Adrenal hypoplasia due to *SAMD9* is discussed in B-2.

Diabetes Mellitus

DM is a condition of chronic hyperglycemia due to the dysregulation of glucose homeostasis. Insulin is the only

hormone that decreases blood sugar, and the pathophysiology of diabetes can be classified into two main groups: insulin deficiency and increased insulin resistance. The major pathogenesis of insulin deficiency is an autoimmune response. A targeted immune response by both T and B cells leads to the destruction of insulin-producing β-cells in the islets of the pancreas. This condition is called T1DM, and some IEI are frequently associated with T1DM, such as APS type 1 (*AIRE*), IPEX (*FOXP3*), *CTLA4*, *LRBA*, and GOF of *STAT1* (22, 31, 35, 63, 103).

One distinctive clinical feature of T1DM associated with IEI is age at onset. Generally, an average age of onset of childhood-onset T1DM is approximately 9 ~ 10 years (104), and onset of T1DM occurs after six months of age. Accordingly, diabetes that develops before six months of age is classified as neonatal diabetes, in which other pathophysiologies, such as congenital malfunction of β-cells, are involved (105). However, several studies have revealed that T1DM associated with IEI frequently develops before six months of age (43, 103). In such patients, T1DM would be the first clinical symptom, and subsequently, immunodeficiency becomes clinically obvious. Accordingly, for T1DM patients whose onset is before six months of age, intensive genetic analysis, including IEI genes, is required.

Some IEI due to DNA repair defects, such as AT and Bloom syndrome, are reported to be associated with DM (106). In such IEI, insulin synthesis is generally maintained, and extremely increased insulin resistance has been suggested to contribute to the development of DM. In AT, lipodystrophy has been suggested as a cause of DM (52), and possibly, in Bloom syndrome, similar mechanisms would be involved, although precise analyses have not yet been reported.

A recent prospective analysis of 39 patients with AT showed a significant increase in HbA1c and fasting glucose levels with age (107). OGTT has good sensitivity for insulin resistance, and 7/39 (17.9%) patients had diabetes. In AT, metformin did not always lead to sufficient glycemic control (107).

ENDOCRINE AND METABOLIC DISORDERS CAUSED BY HCT FOR IEI

Impaired Reproductive Function Caused by HCT

Most IEI are due to defects in genes that are essential for hematopoietic cells to function normally, and supportive therapies, such as immunoglobulin G (IgG) infusions and antibiotics, are occasionally unsatisfactory for long-term outcomes. The only curative approach is the replacement of impaired cells by healthy donor hematopoietic stem cells and allogeneic HCT (allo HCT) (108–110). One of the clinical implications of HCT is the long-term complications that cause chronic and irreversible damage to various organs (111), including the endocrine glands. The major potential causes for long-term complications are conditioning regimens with cytotoxic agents and therapies for GVHD, including glucocorticoids (71).

The purpose of conditioning therapies is to ablate immune cells to allow stable engraftment of donor-derived HSCs. Classically, antineoplastic alkylating agents are used as key drugs in conditioning regimens for HCT. Although recent technical advances in HCT have enabled the reduction of the intensity of the regimen, alkylating agents are still widely used as the central drug (112, 113).

One of the common adverse effects of alkylating agents is germ cell toxicity. The clinical phenotypes of germ cell defects differ between female and male patients. In the ovaries, follicular structures, including oocytes, are essential for the differentiation and maintenance of steroidogenic ovarian cells, granulosa cells, and theca cells. Oocyte depletion due to alkylating agents causes hypogonadism with loss of estrogen and progesterone. In contrast, Leydig cells, which are steroidogenic cells in the testes, are viable in the absence of germ cells. Accordingly, normal androgen synthesis does not indicate the viability of germ cells in the testes, i.e., even after developing secondary sexual characteristics, future fertility of the male subjects is not warranted in IEI patients after HCT.

In contrast to malignant diseases, the conditioning regimen for IEI is generally less toxic, and the complications of HCT for patients with IEI would be different from that of malignant diseases, requiring careful evaluation of the toxicity of alkylating agents. However, evaluating the adverse effects of alkylating agents is not straightforward in IEI. First, the majority of severe IEI cases requiring HCT are male patients because of X-linked genetic diseases. Second, in contrast to hypogonadism, screening for isolated infertility in male patients is difficult, especially during childhood.

A recent report suggested that lower doses of alkylating agents for a reduced intensity conditioning regimen (RIC) would reduce the toxicity, including hypogonadism. A long-term follow-up study of 43 pediatric patients with non-malignant disease after HCT conditioned by RIC regimen revealed that all three postpubertal female patients before HCT resumed normal menstrual cycles post-HCT, and apparent hypogonadism were observed only one (5%) patient (112). A similar outcome was obtained from another cohort conditioned by fludarabine and melphalan based RIC (114). On the other hand, another report suggested that even RIC regimen would be a risk for hypogonadism in female patients (71). Treosulfan is an alkylating agent and increasingly used in recent pediatric practice because of less systemic toxicity than busulphan. By assessing bio markers for potential fertility, such as Anti-Müllerian hormone (AMH) and inhibin B, a treosulfan-based regimen confers a more favorable outcomes for gonadal reserve than fludarabine and melphalan or busulphan/cyclophosphamide based in both sexes (115).

With regard to the gonadal toxicity of alkylating agents, we should be aware that there is no uniformly "safe" or "toxic" alkylating agent dose threshold. Despite a cohort based on malignant diseases in which higher doses of alkylating agents are generally administered, a study of 214 cancer survivors found that despite a negative correlation between cyclophosphamide equivalent dose and sperm concentration, a firm threshold dose

could not be established because azoospermia was observed even at the lowest dose (116).

Another issue for reproduction in IEI patients is age at HCT. Most patients with IEI receive HCT before the reproductive age (108, 112), resulting in limited options for the preservation of fertility before HCT, such as cryopreservation of gonadal tissues. However, at present, cryopreservation of gonadal tissues is considered an experimental technique, and this procedure may only be utilized for carefully selected patients as an experimental protocol (117). Further accumulation of these cases is necessary to elucidate the germ cell toxicity of RIC.

Endocrine and Metabolic Disorders Caused by Prolonged Glucocorticoid Therapy

Long-term administration of glucocorticoids for GVHD after HCT or autoimmune response can cause various clinical problems, such as iatrogenic Cushing's syndrome. The major adverse effects in the metabolic and endocrine systems include glucose intolerance, truncal obesity, and osteoporosis. For these adverse effects, careful and regular monitoring is required (118). Although the clinical presentation of iatrogenic Cushing's syndrome in the pediatric population is similar to that in adults, there are some child-specific features, such as growth retardation. For the management of pediatric patients treated with systemic glucocorticoid therapy, frequent evaluation of linear growth and body weight change by plotting a growth chart is essential (119).

In addition to Cushing's syndrome, patients treated with glucocorticoids are at high risk for adrenal insufficiency. Anti-inflammatory glucocorticoid treatment for IEI suppresses ACTH and CRH production from the pituitary gland and hypothalamus, respectively, decreasing endogenous cortisol synthesis (118). Subsequently, prolonged ACTH suppression leads to adrenal gland atrophy. Atrophic adrenal glands are not capable of promptly and properly synthesizing sufficient endogenous cortisol by abrupt cessation or rapid withdrawal of glucocorticoids, resulting in symptoms of adrenal insufficiency (118).

Furthermore, patients treated with low to medium doses of glucocorticoids are also at high risk for potentially life-threatening acute adrenal insufficiency. The low to medium dose of glucocorticoids suppresses the HPA axis, leading to adrenal atrophy. However, such a dose of glucocorticoid might not sufficiently tolerate physical stresses, such as fever, major injuries, seizure, and frequent vomiting, in which the required volume of glucocorticoid is dramatically increased (118). Physicians should always pay attention to adrenal insufficiency in patients treated with glucocorticoids.

CONCLUSION REMARKS

In summary, we comprehensively reviewed and discussed the current understanding on the clinical features and the pathophysiology of endocrine disorders in IEI. We discussed the two major pathophysiology of endocrinopathy in IEI, autoimmune response and direct effects of the responsible genes, the details of each endocrinopathy, and potential endocrinopathy due to hematopoietic stem cell transplantation. Our expanding understanding on the molecular mechanisms of IEI have shed light on the new aspects of pathophysiology of endocrinopathies. Greater appreciation of these issues will provide valuable insights of the mechanisms underpinning endocrinopathies.

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All authors conceptualized, planned, wrote, and approved the manuscript.

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A20 Haploinsufficiency in East Asia

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A20, encoded by the *TNFAIP3* gene, is a negative regulator of tumor necrosis factor (TNF)-nuclear factor-κB signaling. It was recently demonstrated that A20 haploinsufficiency (HA20), caused by a heterozygous mutation in the *TNFAIP3* gene, can present as an early onset autoinflammatory disease resembling Behçet's disease (BD). In addition to autoinflammatory symptoms, HA20 was also reported to be associated with autoimmune diseases and immunodeficiency. Because the phenotypes associated with HA20 are broad, with different severities observed even among individuals in the same family with identical mutations, it has been assumed that the symptoms of HA20 may depend on genetic background and environmental factors. In this review, we summarize the characteristics of patients with HA20 in East Asia and compare these with patients in other regions, mainly the USA and Europe. Patients with HA20 in East Asia developed recurrent fever more frequently than patients in other regions, but were less likely to develop typical BD symptoms such as skin rashes and genital ulcers. In addition, patients with HA20 in East Asia had low rates of complication with autoimmune diseases and low autoantibody detection rates. While anti-TNF-α agents were the primary treatments for severe HA20 in East Asia, anti-interleukin-1 agents and Janus kinase inhibitors were also administered in other regions. Future studies will need to establish methods for analyzing the pathophysiology of HA20 and determining optimal treatment strategies for each patient.

Keywords: autoimmune disease, autoinflammatory disease, A20 haploinsufficiency, East Asia, *TNFAIP3*

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INTRODUCTION

Behçet's disease (BD) is a chronic inflammatory disease first described by Hulusi Behçet in 1937. BD is characterized by recurrent aphthous stomatitis, skin lesions, uveitis, and genital ulcers (1). Gastrointestinal, cardiovascular, and central nervous system symptoms can also occur in patients with BD. BD is also called the Silk Road disease and has higher prevalence in the Mediterranean, the Middle East, and East Asia compared with other regions. BD shows familial clustering, and thus it has been proposed that genetic predisposition might be a major factor in pathogenesis. Associations

Abbreviations: BD, Behçet's disease; BCR, B-cell receptor; DM, diabetes mellitus; EBV, Epstein–Barr virus; HA20, A20 haploinsufficiency; HCT, hematopoietic cell transplantation; HLA, Human Leukocyte Antigen; IKK, IκB kinase; IL, interleukin; IRF, interferon regulatory factor; ISGFBD, International Study Group for Behçet's disease; ISGs, IFN-inducible-genes; JAK, Janus kinase; NF, nuclear factor; OTU, ovarian tumor; SLE, systemic lupus erythematosus; TBK1, TANK-binding kinase 1; TLR, Toll-like receptors; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor; ZnF, zinc finger.

between BD and human leukocyte antigen (HLA) genes and polymorphisms, especially HLA-B51, are well established. Recent genome-wide association studies (GWAS) reported that polymorphisms in *IL-10*, *IL-23R*, and *IL-12RB2* were associated with risk of developing BD; however, none contributed directly to the onset of BD (2) with high penetrance. In 2016, A20 haploinsufficiency (HA20), caused by heterozygous mutation of the *TNFAIP3* gene, was reported to cause an early-onset autoinflammatory disease presenting with BD-like features such as recurrent aphthous stomatitis, genital ulcers, and gastrointestinal symptoms (3). Since that time, numerous cases with varied clinical presentation have been reported all over the world, especially in East Asia including China and Japan. Several studies have investigated the function of A20, the pathophysiology of HA20, and optimal treatment strategies. Furthermore, patients with HA20 can present with both autoinflammatory BD-like symptoms as well as autoimmune symptoms and/or immunodeficiency. Variation in symptoms, disease severity, and response to treatment occurs even in individuals within a single family carrying identical mutations. To describe the characteristics of HA20 in East Asia and future prospects for treatment of HA20, we reviewed the published literature on HA20 in East Asia and other regions, focusing on disease manifestations, complications, treatments, and distribution of *TNFAIP3* mutations.

FUNCTION OF A20

A20 is encoded by the *TNFAIP3* gene located on chromosome 6q23.3. A20 is a negative regulator of tumor necrosis factor (TNF)-nuclear factor (NF)-κB signaling. In the NF-κB signaling pathway, signals transmitted from TNF-α, interleukin (IL)-1 family members, Toll-like receptors (TLRs), B-cell receptors (BCRs), and T-cell receptors result in ubiquitination of factors in each pathway, NF-κB activation, and production of inflammatory cytokines (4). A20 has an ovarian tumor (OTU) domain in the N-terminal region and seven zinc finger (ZnF) domains in the C-terminal region. The OTU domain deubiquitinates K63 polyubiquitin chains from receptor interacting protein 1, TNF receptor-associated factor (TRAF) 6, and IκB kinase (IKK) γ (5–7) resulting in suppression of signaling. In addition, the ZnF4 domain has E3 ligase function and supports polyubiquitination with K48 polyubiquitin chains, inducing degradation by the proteasome (6). The ZnF7 domain binds the TNF receptor complex *via* linear polyubiquitin chains and inhibits the formation of the linear ubiquitin chain assembly complex and the IKK complex (8). Posttranslational modifications of A20 have suppressive effects on this pathway: for instance, IKKβ-mediated serine phosphorylation near the ZnF domains (notably at Ser381) promotes A20-mediated cleavage of K63-linked polyubiquitin chains and enhances the inhibitory activity of A20 (9). These functions of A20 contribute to inhibition of NF-κB signaling from each receptor.

A20 also functions to regulate the interferon regulatory factor (IRF) pathway following pathogen recognition. In this pathway, the pattern recognition receptors retinoic acid-inducible gene I and melanoma differentiation-associated protein 5 recognize viral nucleic acids and activate mitochondrial antiviral signaling protein and TRAF3. Subsequently, they combine with TANK-binding kinase 1 (TBK1) and IKKi and are activated by ubiquitination with K63 polyubiquitin chains. Activation of these kinases results in phosphorylation, dimerization, and nuclear translocation of the transcription factor IRF3, followed by transcriptional activation of the type 1 interferon (IFN) gene. Type 1 IFN binds to the IFN-α/β receptor, activates Janus kinase (JAK)-signal transducer and activator of transcription signaling, and induces transcription of IFN-inducible-genes (ISGs). A20 inhibits the IRF pathway and IFN responses by cleaving K63 polyubiquitin chains from TBK1/IKKi (4). In patients with HA20, disrupted suppression of signal transduction results in increased production of inflammatory cytokines and type 1 IFNs followed by autoinflammatory symptoms.

PATIENTS WITH HA20 IN EAST ASIA AND OTHER REGIONS

We summarized the literature on patients with HA20 resulting from *TNFAIP3* mutations starting from the first report of this disease in 2016 until August 2021. A total of 20 studies in East Asia and 15 studies in other regions, mainly the USA and Europe, were published during this period (10–44). Case reports from East Asia were analyzed separately from those in other regions. We focused on major BD-like symptoms, development of autoimmune diseases, non-autoimmune complications, and treatments administered. The following East Asian patients with HA20 were excluded: one patient's gender was not stated, three patients did not have detailed symptom information, and two of these three patients were not investigated for autoimmune diseases and/or autoantibodies. A single patient described by multiple studies was counted as one case. The prevalence of HA20 symptoms was compared between East Asia and other regions using Fisher's exact test in Prism 7 (GraphPad Software, San Diego, CA, USA). Values of $p < 0.05$ were considered statistically significant.

Table 1 shows the symptoms of HA20 patients in East Asia and other regions. A total of 74 patients in 39 families were affected by HA20 in East Asia, while a total of 51 patients in 23 families were affected by HA20 in other regions. The age of onset ranged from neonatal to around 30 years in both groups. Thus, the age of onset of HA20 appears to be younger than that of BD.

LOCATIONS OF *TNFAIP3* MUTATIONS IN EAST ASIA

The domain structure of A20 and sites of mutations in *TNFAIP3* among East Asian patients with HA20 are shown in **Figure 1**. A20 mutations in patients with HA20 in East Asia were widely distributed from the OTU to the ZnF7 domains. Most mutations were truncating and included frameshift mutations, nonsense mutations, splice site mutations, and large deletions. However, some missense mutations were also reported. Three missense mutations that were judged as pathogenic *via* functional analyses

TABLE 1 | The comparison of symptoms and treatment between East Asia versus other countries.

	Countries		*p*-value
	East Asia	**without East Asia**	
Counts of family	39	23	
Counts of Patients	74	51	
Number of male patients: n/total patients (%)	35/73 (47.9%)	15/51 (29.4%)	
Range of onset age	neonatal period to 32 y	2 mo to 29 y	
Symptoms: n/total patients (%)			
Recurrent stomatitis	57/71 (80.2%)	42/51 (82.6%)	0.819
Cutaneous lesions	23/71 (32.4%)	27/51 (52.9%)	0.026
Ocular symptoms	3/71 (4.2%)	5/51 (9.8%)	0.277
Genital ulcers	27/71 (38.0%)	34/51 (66.7%)	0.003
Arthritis	23/71 (32.4%)	22/51 (43.1%)	0.257
Abdominal symptoms	40/71 (56.3%)	28/51 (54.9%)	>1.000
Cardiovascular lesion	2/71 (2.8%)	5/51 (9.8%)	0.128
Central nervous system symptoms	3/71 (4.2%)	2/51 (3.9%)	>1.000
Pathergy	2/71 (2.8%)	4/51 (7.8%)	0.235
Recurrent fever	50/71 (70.4%)	19/51 (37.3%)	<0.001
Criteria of ISGFBD 1990 fulfil/total patients (%)	18/71 (25.4%)	19/51 (37.3%)	0.169
Number of autoimmune diseases and/or autoantibodies	21/72 (29.2%)	30/51 (58.8%)	0.002
Development of autoimmune diseases	Autoimmune thyroid disease, AIH, SLE, ALPS-U, PsA, Detection of autoantibodies	Autoimmune thyroid disease, SLE, ITP, type 1 DM, PsA, Detection of autoantibodies	
Symptoms that may be associated with HA20 without autoimmune diseases	IgA vasculitis, CH, IP, Lymphadenitis, Nephrotic syndrome, Aseptic meningitis, DD, MAS, HL, Craniopharyngioma, BCG dermatitis, Chronic active EBV infection	IgA vasculitis, CH, IP, Lymphadenitis, Cerebral infraction, Pancytopenia, IgG2 and IgG4 deficiency, Recurrent infection, Chronic active EBV infection, KD like Coronary vasculitis	
Treatment: Number of used patients (%)			
Colchicine	18 (24.3%)	18 (35.3%)	
Steroid	34 (45.9%)	19 (37.3%)	
Anti-TNF-α agents (infliximab, adalimumab, etanercept)	21 (28.4%)	14 (27.5%)	
Anti-IL-1 agents (anakinra, canakinumab, rilonacept)	1 (1.4%)	10 (19.6%)	
Tocilizumab	3 (4.1%)	3 (5.9%)	
Rituximab	1 (1.4%)	2 (3.9%)	
JAK inhibitor agents (tofacitinib, baricitinib)	1 (1.4%)	6 (11.8%)	
Hematopoietic cell transplantation	1 (1.4%)	2 (3.9%)	
Sirolimus	0 (0.0%)	1 (2.0%)	
NSAIDs	4 (5.4%)	1 (2.0%)	
Methotrexate	12 (16.2%)	7 (13.7%)	
Cyclophosphamide	3 (4.1%)	1 (2.0%)	
Cyclosporine A	6 (8.1%)	3 (5.9%)	
Tacrolimus	2 (2.7%)	1 (2.0%)	
Mycophenolate mofetil	4 (5.4%)	4 (7.8%)	
Azathioprine	3 (4.1%)	9 (17.6%)	
Mesalazine	12 (16.2%)	2 (3.9%)	
Thalidomide	8 (10.8%)	3 (5.9%)	
Hydroxychloroquine	3 (4.1%)	3 (5.9%)	
Dapsone	0 (0.0%)	2 (3.9%)	
Cimetidine	2 (2.7%)	0 (0.0%)	
Iguratimod	1 (1.4%)	0 (0.0%)	
Mizoribine	1 (1.4%)	0 (0.0%)	
IVIG	1 (1.4%)	2 (3.9%)	

AIH, Autoimmune hepatitis; ALPS-U, autoimmune lymphoproliferative syndrome undefined; BCG, Bacille de Calmette et Guérin; CH, chronic hepatis; DD, developmental disability; DM, diabetes mellitus; EBV, Epstein-Barr Virus; HL, Hodgkin lymphoma; Ig, immunoglobulin; IL, interleukin; IP, interstitial pneumonia; ISGFBD, International Study Group for Behçet's disease; ITP, idiopathic thrombocytopenic purpura; IVIG, intravenous immunoglobulin; JAK, Janus kinase; KD, Kawasaki disease; MAS, Macrophage activation syndrome; NSAID, nonsteroidal anti-inflammatory drug; PsA, psoriatic arthritis; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor.

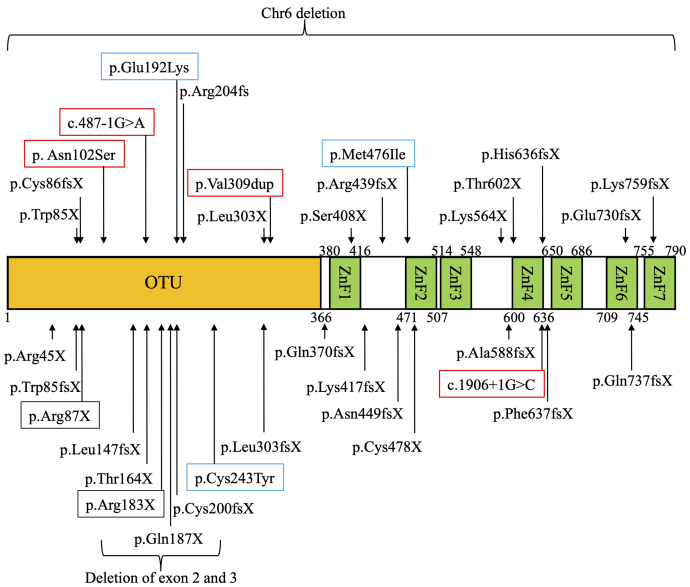

FIGURE 1 | Domain structure of A20 and locations of *TNFAIP3* gene mutations in East Asian patients with A20 haploinsufficiency (HA20). Mutations of *TNFAIP3* reported in East Asia are indicated with arrows on the domain structure of A20. Three mutations whose pathogenic functions were evaluated by *in vitro* functional analysis are shown in blue squares. Four mutations whose pathogenic functions were not evaluated by *in vitro* functional analysis are shown in red squares. The mutations overlapping between different families are shown in black squares.

are shown in blue squares. *TNFAIP3* Cys243Tyr (45) and Glu192Lys (18) were reported in Japan, while Met476Ile (20) was reported in China. Among previously reported mutations, the pathogenic significance of four potential splice site mutations or duplication mutations (shown in red squares) has not been evaluated using functional analyses. One of these mutations was reported in Japan (16), while four were reported in China (25, 27). Among the mutations in East Asia, Arg183X was found in two families (22, 27). The only mutation overlapping between East Asia and other regions was Arg87X, which was found in one family in each case (21, 42).

CLINICAL SYMPTOMS OF HA20

Table 1 shows the symptoms of patients with HA20 in East Asia and other regions. Recurrent stomatitis was the most common symptom in both groups of patients with a prevalence of 80.2% and 82.6%, respectively. In East Asia, the next most common symptoms of HA20 were recurrent fever, abdominal symptoms, genital ulcers, and skin rashes. In other regions, these symptoms were also common, albeit with different frequencies. Recurrent fever was significantly more common among East Asian patients compared with those in other regions (70.4% *vs.* 37.3%, $p < 0.001$). Interestingly, the major BD-like symptoms of genital ulcers and skin rash, including erythema nodules, folliculitis-like eruption, and thrombophlebitis, were more common among patients with HA20 in other regions compared with East Asia. There was no significant difference in the proportion of HA20 patients in East Asia and other regions who fulfilled the International Study Group for Behçet's disease (ISGFBD) 1990 criteria (46). However, there was a trend toward higher frequency of meeting these criteria in other regions compared with East Asia (25.4% *vs.* 37.3%, $p = 0.169$).

COMPLICATIONS ASSOCIATED WITH HA20

HA20 has been reported to often lead to autoimmune diseases such as systemic lupus erythematosus (SLE) and autoimmune thyroid disease. We compared the autoimmune disease development rate and/or autoantibody positive rate in East Asia and other regions and found that these rates were significantly lower in East Asia (**Table 1**). Non-autoimmune complications were diverse and included IgA vasculitis, chronic hepatitis, and nephrotic syndrome. One patient with HA20 in East Asia developed Hodgkin lymphoma (10). Interestingly, persistent Epstein-Barr virus (EBV) infection occurred in patients with HA20 in both East Asia and other regions (20, 40). Furthermore, some patients in other regions developed immunodeficiency symptoms such as IgG2 and IgG4 deficiency, pancytopenia, and recurrent infection (30, 40, 41).

TREATMENT OF HA20

The number of patients with HA20 treated with medications and administration rates in East Asia and other regions

are shown in **Table 1**. In both groups of patients, colchicine, systemic corticosteroids, disease-modifying drugs, and molecular targeted therapies were relatively commonly administered. In East Asia, anti-TNF-α agents (infliximab, adalimumab, and etanercept) were administered in most severe cases, while anti-IL-6 agents (tocilizumab) were also administered in some cases (10, 13, 16, 21, 24). Only one patient was treated with anti-IL-1 agents (canakinumab) in East Asia (47). In other regions, patients with severe HA20 who did not respond to anti-TNF-α agents were commonly treated with anti-IL-1 agents (anakinra, canakinumab, and rilonacept), anti-IL-6 agents, and JAK inhibitors (tofacitinib and baricitinib) (30, 32, 35, 36, 38–40). Rituximab was administered in a few patients with nephrotic syndrome or autoimmune diseases including SLE in East Asia and other regions (10, 31, 41). In addition, allogeneic hematopoietic cell transplantation (HCT) was also performed for one refractory patient in East Asia and one refractory patient in other regions (31, 47), and autologous HCT was performed for one patient in other regions (30).

DISCUSSION

This review summarizes the current status of HA20, including regional differences in clinical features, evaluation of the pathogenic significance of *TNFAIP3* variants, and treatment strategies.

We found that the proportions of patients with typical BD symptoms, such as skin rash and genital ulcers, and of patients who met the ISGFBD diagnostic criteria for BD, tended to be lower in East Asia compared with other regions. Additionally, the proportions of patients who were autoantibody-positive and/or who were developed autoimmune diseases were also significantly lower in East Asia compared with other regions. A multicenter study in Japan demonstrated that increased frequencies of double-negative T cells and follicular T cells contributed to the development of autoimmune diseases in patients with HA20 (48). Differences in autoimmune disease development rates between regions might be related to immunological differences associated with racial background. In addition, activation of type 1 IFN was reported to cause autoimmune diseases (49). HA20 patients in other regions were reported to show overexpression of ISGs (36, 38, 40, 43), while only one patient in East Asia showed elevated ISG expression (47). In this study, it is unclear whether the overexpression of ISGs is associated with regional differences in autoimmune disease because few patients were investigated for ISG expression. Future work should investigate the relationship between the expression of ISGs in HA20 and the development of autoimmune diseases. There are insufficient data to account for regional differences in the clinical features of HA20. Variation in the clinical features of HA20 were not completely explained by differences in mutation sites. It has been reported that musculoskeletal disorders were significantly more frequent among patients with mutations disrupting the ZnF domain of A20, whereas other symptoms were not affected by mutation site (50). Because genotype-phenotype correlations have not been demonstrated in HA20, variation in clinical features might be affected by other modifier genes and/or environmental factors. Some HA20 patients present with immunodeficiency symptoms such as hypogammaglobulinemia and persistent EBV infection (20, 30, 40). It was reported that mice with selective loss of A20 in B cells had fewer memory B cell and diminished levels of IgG1 and IgG3 (51). Moreover, in HA20 patients, it was suggested that over-activation of NF-κB signaling may cause T cell exhaustion and senescence and thereby decrease naïve Th cell frequency and facilitate persistent EBV infection (48). The composition of lymphocyte subsets may also be affected by age. We were unable to analyze age of onset in patients with HA20 because age of onset in some patients were not stated. Future studies will need to conduct detailed analyses of lymphocyte subsets in patients with HA20 and immunodeficiency to clarify the underlying pathophysiology.

Most HA20 mutations are truncating and include nonsense, frameshift, and splice site mutations. Three missense mutations have been reported in East Asia: *TNFAIP3* Glu192Lys, Cys243Tyr, and Met476Ile. While these missense mutations have demonstrated pathogenicity in *in vitro* functional analyses (18, 52), the pathogenic significance of other variants, such as p.Asn102Ser, c.487-1G>A, and p.Val309dup, has yet to be demonstrated *via* functional analyses. In particular, the frequency of the Asn102Ser allele is 0.01394 in East Asian according to the Genome Aggregation Database (gnomAD) dataset v.2.1.1 (https://gnomad.broadinstitute.org). Because HA20 is an autosomal dominant disease with high penetrance, *in vitro* functional analysis is essential to demonstrate that this variant with high allele frequency represents a pathogenic mutation. The analysis of NF-κB reporter gene activity has been commonly used as *in vitro* functional analysis to demonstrate the pathological significance of *TNFAIP3* variants, especially for truncating mutations (3, 10). Furthermore, it was reported that disruption of the inhibitory effects of *TNFAIP3* missense variants could be more sensitively distinguished from wild type *TNFAIP3* function by analyzing NF-κB reporter gene activity in response to TLR or BCR signaling (18, 52). However, some missense variants did not show significant difference from wild type *TNFAIP3* using the methods mentioned above (18). One must carefully interpret whether such variants represent low-penetrance mutations, polymorphisms that contribute mildly to the development of HA20, or polymorphisms that contribute to other diseases. *TNFAIP3* Phe127Cys (rs2230926) is a single-nucleotide polymorphism associated with development of autoimmune diseases such as rheumatoid arthritis, SLE, and type 1 diabetes mellitus (DM) (53, 54). However, this variant showed no significant disruption of the inhibitory effects of A20, and it has not been evaluated as a pathogenic mutation for onset of HA20 in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). It cannot be concluded that *TNFAIP3* variants identified by comprehensive genetic analysis are pathogenic based solely on allele frequencies and patient symptoms; validation by functional analysis is required. Furthermore, more sensitive and specific methods for functional analysis of *TNFAIP3* variants of unknown significance are required for future studies.

There is no standard treatment protocol for HA20. Various treatments are used in both East Asia and other regions, including colchicine, systemic corticosteroids, disease-modifying drugs, and molecular targeted therapies. The clinical severity of HA20 varies from mild to severe; responses to treatment also vary significantly. It has been reported that colchicine is effective to some extent in mild cases of HA20 (3, 10, 30, 37). In severe cases with poor response to colchicine, biological drugs are administered such as anti-TNF-α agents, anti-IL-1 agents, and anti-IL-6 agents. The rationale for such interventions is based on the increased production of proinflammatory cytokines such as TNF-α, IL-1β, IL-18, and IFN-γ-induced protein 10 in sera and stimulated peripheral blood mononuclear cells from patients with HA20 (3, 10). These therapies are effective in suppressing systemic inflammation in patients with other diseases. Anti-IL-1 agents were administered to one patient with HA20 in East Asia and were not effective; however, these agents were reported to be effective in some patients in other regions (3, 30, 35, 39, 40). Anti-IL-6 agents have been used less frequently to treat HA20 than anti-TNF-α and anti-IL-1 agents, but have been reported to be effective in a subset of patients (32). The efficacy of anti-IL-6 agents may relate to elevation of serum IL-6 during the active phase of disease (10). The possibility of the secondary ineffectiveness of biological drugs due to production of antibodies for them, especially anti-TNF-α agents, should be considered because severe HA20 patients complicated with autoimmune diseases are expected to have both autoinflammatory conditions and increased antibody production. Among molecular targeted therapies, JAK inhibitors were reported to be effective in patients with HA20 whose ISG expression was elevated in the active disease phase (38). Thus, administration of JAK inhibitors may become a more prominent treatment strategy. Although it has been conducted in only two patients with HA20 to date, HCT was effective in patients resistant to various treatments including several biological agents and led to remission of their inflammatory symptoms (31, 47). By contrast, a patient who received autologous HCT because of central nervous system vasculitis relapsed after 18 months of remission and various immunosuppressive agents were reinitiated (30). The first case of allogeneic HCT was a 14-year-old English boy. He had complex clinical features including development of insulin-dependent DM, cell depletion, hepatitis, enteropathy, and interstitial lung disease. Because his symptoms were refractory to treatment with prednisolone, sirolimus, tacrolimus, infliximab, and rituximab, he received HCT. Following HCT, he entered complete remission from all autoimmune disorders except DM. The second case was a 6-year-old Japanese boy. He had frequent fever, arthritis, psoriasis, aortic regurgitation, bowel disease, and genital ulcers. His symptoms were refractory to treatment with immunosuppressive drugs including prednisolone, cyclosporine, tocilizumab, anti-TNF-α agents, canakinumab, and tofacitinib. He underwent HCT and his autoinflammatory symptoms improved. However, some of his symptoms, such as severe aortic regurgitation and adrenal insufficiency, persisted because of long term administration of steroids. HCT might be an effective strategy for patients with treatment resistant HA20. However, it should be note that not all symptoms improve following HCT and that pre-existing organ damage will remain, potentially leading to symptom relapse. Because HA20 presents with various symptoms and severity, information on more cases is needed to establish appropriate treatment strategies.

CONCLUSION

We summarized the symptoms and treatments of patients with HA20 in East Asia and other regions. Patients in East Asia had significantly higher rates of recurrent fever and lower rates of typical BD symptoms including genital ulcers and skin rashes. Patients in East Asia were less likely to have autoimmune disease complications. Differences between patients with HA20 in East Asia and other regions may relate to variation in the frequencies of other modifier genes and/or environmental factors. Future studies will need to establish functional analysis methods to validate the pathogenicity of *TNFAIP3* variants to enable rapid diagnosis of HA20 and development of appropriate treatment strategies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Creating Awareness for Primary Immunodeficiencies in Japan

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Primary immunodeficiency (PID) is primarily characterized by susceptibility to infectious diseases. In addition, patients with some type of PID are prone to develop autoimmune, autoinflammatory, or malignant diseases. Therefore, the term, inborn errors of immunity (IEI), has been more used rather than PID. In recent years, the number of diseases which belong to PID has been increasing. There were approximately 110 diseases in the report of International Union of Immunological Societies in 1999. Since then, the number increased to 430 diseases in the latest IUIS report in 2019. We conducted PID nationwide survey in Japan for 3 times in the last 15 years. These studies were focused on incidence and complications of PID, the clinical course of viral infection, and methods to prevent infectious diseases in PID patients. For the awareness of PID, it is essential to know the general and fundamental information of PID patients. Needless to say, we need it to offer appropriate medical services for PID patients. Moreover, chances to provide answers to the questionnaires and seeing the results of the analysis should contribute to the awareness of PID among doctors. In this review, I am going to summarize the results of 3 nationwide survey in Japan, and pick up interleukin-1 receptor-associated kinase 4 (IRAK4) deficiency as an example for creating awareness for its appropriate management.

Keywords: primary immunodeficiency, inborn errors in immunity, nationwide survey, interleukin-1 receptor-associated kinase 4 (IRAK-4), questionnaire research

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NATIONWIDE SURVEY OF PID PATIENTS IN JAPAN 2007

We conducted a nationwide questionnaire survey in 2007 to clarify the incidence and the clinical characteristics of PID in Japan (1). The study was conducted according to the nationwide epidemiological survey manual of patients with intractable disease (2nd edition 2006, Ministry of Health, Labour, and Welfare of Japan) (2). Questionnaires were sent to 1224 pediatric departments and 1670 internal medicine departments of hospitals. Primary questionnaire was aimed to reveal the prevalence of PID in Japan, and the clinical data of the patients were obtained by the secondary questionnaire. The response rates for the questionnaire survey from the pediatric departments and the departments of internal medicine were 55.5% and 20.1%, respectively. We found that the number of PID patients was estimated to be 2900 (95% confidence interval: 2300-3500), and the prevalence of PID in Japan was 2.3/100,000 inhabitants. The prevalence was equivalent to that reported from Singapore (3) and Taiwan (4) (2.7/100,000 and 0.8-2.2/100,000, respectively), but was lower than that reported from Europe, 4.4/100,000 in France and 6.8 in Norway (5, 6).

By the secondary questionnaire, we found that predominantly antibody deficiency was the most common (40%), followed by congenital defects of phagocyte number, function or both (19%) among the PID disease categories. Bruton's tyrosine kinase (BTK) deficiency was found to be the most common PID in Japan, and accounted for a higher rate (14.7%) than in European countries (5.9%) (7). On the other hand, the prevalence of common variable immunodeficiency (CVID) was low (11.0%). One of the reasons why the prevalence of BTK deficiency was high and that of common variable immunodeficiency (CVID) was low in this study compared with that reported from Europe might be the high questionnaire response rate from pediatric departments compared with that from department of internal medicine in this study. The prevalence of chronic granulomatous disease (CGD) (11.9%) was also found to be the common PID in Japan. Although the genetic background of Japanese CGD is different from that of European countries, the prevalence seems to be similar (8).

This study also revealed that malignant diseases were observed in 2.7% of PID patients, as previously published (1). Epstein–Barr virus-related or unrelated lymphoma, and leukemia were found predominantly. CVID, Wiskott–Aldrich syndrome, and ataxia telangiectasia were frequently associated with such malignant diseases with the percentages of 7.0%, 8.1% and 1.3%, respectively. Complication of immune-related diseases were also found in 8.5% of PID patients, especially in patients with autoimmune lymphoproliferative syndrome and immune dysregulation, polyendocrinopathy, enteropathy X-linked. Interestingly, they were also observed in nuclear factor kappa B (NF-κB) essential modulator (NEMO) deficiency patients. The main immune-related complication was immune related bowel disease, such as inflammatory bowel disease and autoimmune enteritis, which was found in 3.6% of PID patients. Other unique complications of PID patients were immune related and non-immune-related endocrine diseases (9). Some of these complications were clearly associated with the genetic cause of PID, but others were difficult to explain the pathogenesis in connection with it. The most common endocrine complication was hypoparathyroidism which was found in1.6% of total PID patients. Fourteen out of 15 PID patients complicated with hypoparathyroidism had DiGeorge syndrome. The other patient had autoimmune polyendocrinopathy-candidiasis-ectodermal syndrome (APECED). Non-autoimmune hypothyroidism, autoimmune hypothyroidism, type 1 diabetes were found in 0.8%, 0.5% and 0.9%, respectively. The incidence of such endocrine complications in PID patients was higher than that in general population.

NATIONWIDE SURVEY OF PID PATIENTS IN JAPAN 2013

The severity and clinical course of common viral infections in PID patients had not been investigated in a large cohort of PID patients. We conducted the nationwide questionnaire study in 2013 to reveal the clinical course of respiratory syncytial (RS) virus, rotavirus, influenza virus, and varicella-zoster virus infections in PID patients (10). These 4 infectious diseases were chosen because the pathogens are usually identified with sufficient accuracy by using rapid antigen detection test (RS virus, rotavirus and influenza virus) or the correct diagnosis can be made by clinical manifestation (varicella). We found that 51 of 910 (5.6%) PID patients were hospitalized for the treatment of one of the 4 viral infectious diseases. Patients with cellular immunodeficiency (combined immunodeficiency and combined immunodeficiencies with associated or syndromic features) shared 32.7% of total PID patients. As expected, the ratios of patients with cellular immunodeficiency among hospitalized PID patients for the treatment of RS virus, Rotavirus, influenza and varicella-zoster virus infection were 53.3%, 40.0%, 35.0% and 60.0%, respectively. Patients with cellular immunodeficiency tended to have a longer duration of hospital stay, increased ratio of needs for mechanical ventilation, and/or death from these infectious diseases (10). These findings support the pivotal role of cellular immunity against these viral infections, and warrant the application of palivizumab to PID patients, with cellular immunodeficiency, in particular.

NATIONWIDE SURVEY OF PID PATIENTS IN JAPAN 2018

We conducted an additional nationwide survey in Japan in 2018, focusing on protective measures for PID patients against infectious diseases (11). In this study, a total of 1,307 patients were reported. Therefore, the prevalence of PID was estimated to be 2.2 patients per 100,000 population, which was similar to the result of nationwide survey of 2007. The most common disease category was autoinflammatory disorders (25%), followed by antibody deficiencies (24%) and congenital defects of phagocyte number or function (16%) It was surprising because the most common disease category was predominantly antibody deficiency, followed by congenital defects of phagocyte number, function or both in the 2007 survey. Among autoinflammatory syndrome, the most common disease was familial Mediterranean fever. The increased ratio of familial Mediterranean fever might be due to the spreading knowledge of familial Mediterranean fever, which had previously been believed to be rare in Japan (12).

In this study, we first focused on the vaccination for the PID patients. One of the crucial findings was that a significant number of patients received contraindicated vaccines (12). Severe combined immunodeficiency (SCID) is not routinely screened at birth in Japan. These vaccines were administered mainly because the patients were not diagnosed as PID by the time of vaccination. Actually, forty-three percent of PID patients for whom bacillus Calmette-Guérin (BCG) vaccination was contraindicated were inoculated with BCG, and 14% of the inoculated patients developed BCG infections. BCG infections developed predominantly in patients with CGD and Mendelian susceptibility to mycobacterial diseases (MSMD). BCG is scheduled for children during the first year of life in Japan,

and Japan Pediatric Society recommends BCG inoculation between 5 and 7 months after birth. The method to diagnose very early in life before the vaccination age should be established for patients with CGD and MSMD in addition to the SCID patients. Other statistical analysis regarding methods of prevention against infectious diseases is ongoing.

PREVENTION OF INVASIVE BACTERIAL INFECTION IN PATIENTS WITH INTERLEUKIN-1 RECEPTOR-ASSOCIATED KINASE 4 DEFICIENCY

Among PID which is comprised of more than 430 diseases, IRAK4 deficiency is one of the PID to which we should create awareness. We have compiled data of all Japanese IRAK4 deficient patients independent of the nationwide studies. IRAK4 deficiency patients are at increased risk of having invasive bacterial infection, predominantly caused by pneumococcus, *Staphylococcus aureus*, streptococci, and *Pseudomonas aeruginosa*. Especially, pneumococcal meningitis is the most common serious infection in Japanese patients (13). The disease cannot be diagnosed unless the doctors have an awareness of the disease, because it lacks the specific laboratory findings. IRAK4 is a molecule, similar to myeloid differentiation primary response gene 88 (MyD88), which mediates signal transduction from most Toll-like receptors (TLR), IL-1 receptor, and IL18 receptor. The clinical manifestation of MyD88 deficiency is very similar to that of IRAK4 deficiency. For these diseases, the establishment of preventive method against invasive bacterial infection is of extreme importance, because the mortality rate of the invasive bacterial infection is as high as approximately 50%, and intriguingly, the patients become less susceptible to these infectious pathogens after infancy or early childhood. We investigated serotype-specific opsonophagocytic activity in serum from IRAK4 deficient patients before and after the vaccination of conjugated or non-conjugated pneumococcal vaccine to determine whether anti-pneumococcal vaccination was effective or not (14). We found that IRAK4 deficiency patients produced protective levels of anti-pneumococcal antibody after the vaccination. Therefore, pneumococcal vaccination schedules are recommended.

On the other hand, pneumococcal vaccination cannot cover all pneumococcal serotypes. Conjugated vaccine contains only 13, and non-conjugated vaccine contains 23 polysaccharide antigens. Invasive pneumococcal infection caused by non-vaccine strain is increasing after the application of conjugated pneumococcal vaccine in Japan (15, 16). We therefore recommend the use of penicillin and sulfamethoxazole-trimethoprim at least until 8 years of age, in addition to the conjugated vaccine followed by non-conjugated pneumococcal vaccine for the prophylaxis of invasive pneumococcal infection (14). Moreover, especially during the infantile period and early childhood, regular immunoglobulin therapy may be required. This strategy to prevent invasive bacterial infection should also be applied to MyD88 deficiency.

SUMMARY

General and fundamental information is necessary for the appropriate diagnosis and management of PID patients. Genetic diagnosis is essential for the diagnosis of PID. We have to take into account any difference of clinical manifestation based on the genetic background or ethnic groups, which was typically found in familial Mediterranean fever. Nationwide study and national registration system may further contribute to the accumulation of information how to assess PID patients. It has been reported that neonatal screening of SCID plays an important role for the early diagnosis and fair outcome after hematopoietic stem cell transplantation. We are expecting the establishment of screening system for patients with CGD and MSMD, because the patients might receive BCG which cause severe and life-threatening BCG infection in Japan. PID is a rare disease. To offer best medical services to PID patients, we have to offer an easy access to updated information regarding PID for doctors.

In conclusion, recent nationwide questionnaire surveys which were carried out 3 times in the last 15 years clarified the prevalence and clinical characteristics of PID. And the importance of nationwide study of specific disorders was shown by considering IRAK4 deficiency as an example. Furthermore, we clarified complications of PID, problems of vaccination in PID patients and needs for the newborn screening.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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CASE REPORT
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Case Report: Analysis of Preserved Umbilical Cord Clarified X-Linked Anhidrotic Ectodermal Dysplasia With Immunodeficiency in Deceased, Undiagnosed Uncles

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Family history is one key in diagnosing inborn errors of immunity (IEI); however, disease status is difficult to determine in deceased relatives. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is one of the hyper IgM syndromes that is caused by a hypomorphic variant in the nuclear factor kappa beta essential modulator. We identified a novel *IKBKG* variant in a 7-month-old boy with pneumococcal rib osteomyelitis and later found that his mother has incontinentia pigmenti. Genetic analysis of preserved umbilical cords revealed the same variant in two of his deceased maternal uncles. Analysis of preserved umbilical cord tissue from deceased relatives can provide important information for diagnosing IEI in their descendants.

Keywords: preserved umbilical cord, hyper IgM syndrome, anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID), NEMO, case report

INTRODUCTION

Family history is one of the most important items of the 10 Warning Signs of Primary Immunodeficiency Diseases for the prediction of primary immunodeficiency diseases (1), now the term inborn errors of immunity (IEI) is used instead in the International Union of Immunological Societies classification (2). However, IEI is often not diagnosed, even when suspected in deceased relatives, because diagnoses and/or diagnostic tools were not available in previous generations. In Japan, preserved umbilical cords are stored at home as a memento of a birth, and Japanese maternity clinics and hospitals customarily present such tissue as a gift to parents. Diagnostic use of dried umbilical cord has been reported for congenital infections by cytomegalovirus (3) and rubella (4), neonatal enterovirus infection (5), and transient abnormal myelopoiesis (6); however, preserved umbilical cord has never been used to diagnose IEI.

X-linked (XL) anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) is a rare IEI. XL-EDA-ID is based on a hypomorphic variant of *IKBKG* (on Xq28), which encodes the nuclear factor kappa beta (NF-κB) essential modulator (NEMO). An *IKBKG* variant is also associated with

incontinentia pigmenti (IP) in females. Although the typical variant in IP (deletion of exons 4-10, accounting for >80% of cases) is lethal in males (7), hypomorphic variants can result in surviving males and various clinical phenotypes, including ectodermal dysplasia presenting with aberrant development of hair (hypotrichosis or atrichosis), teeth (hypodontia or anodontia with conical incisors), and eccrine sweat glands (hypohidrosis or anhidrosis), recurrent severe infections, osteopetrosis, lymphedema, and colitis (8–10). Not all of these are relevant; however, several variants have genotype-phenotype correlations (11). Herein, we describe a 7-month-old boy with a novel variant of *IKBKG* and acute rib osteomyelitis caused by *Streptococcus pneumoniae*. Two of his deceased maternal uncles were successfully diagnosed by XL-EDA-ID analysis of their preserved umbilical cords, which had been stored for 40 years.

CASE REPORT

A 7-month-old boy presented to our emergency department with a mass in his left anterior chest and a fever of 2 days' duration. The patient had delayed umbilical cord separation at 6 weeks of age. He had been vaccinated successfully, without adverse reactions, in accordance with the Japanese national immunization program, and had received one dose of Bacille Calmette-Guérin vaccine and three doses of 13-valent pneumococcal conjugate vaccine, *Haemophilus influenzae* type b vaccine, hepatitis B vaccine, and pentavalent rotavirus vaccine. The patient was born to non-consanguineous parents, and he had been thriving without growth failure. Family history was significant for two maternal uncles who had died at ages 4 and 7 months (**Figure 1A**). The older uncle had persistent refractory diarrhea. The maternal grandmother mentioned that both boys had hypogammaglobulinemia; however, their medical records from 40 years previously had been discarded.

On physical examination, the vital signs were a body temperature of 38.2°C, heart rate of 181 beats/min, respiratory rate of 48 breaths/min, oxygen saturations of 98% in room air, and blood pressure of 123/77 mm Hg. He appeared well. Examination of his left anterior chest wall revealed a firm, non-fluctuant subcutaneous mass, 5 cm in diameter, redness of the overlying skin, and no tenderness. He had dry skin and features of ectodermal dysplasia, including sparse hair and eyebrows, a depressed nasal bridge, no eruption of the deciduous teeth, and

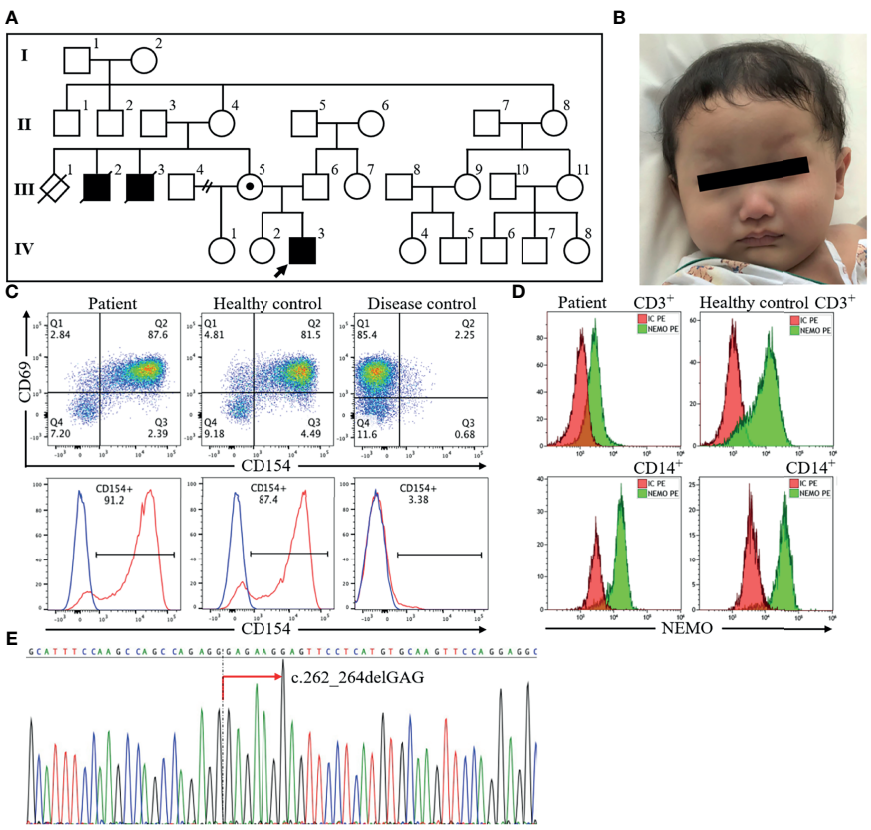

FIGURE 1 | The profile of the index patient. The patient, a 7-month-old boy indicated by an arrow in the pedigree diagram **(A)**, had sparse hair and eyebrows and a depressed nasal bridge **(B)**. Flow cytometric analysis of peripheral blood revealed normal CD40 ligand (CD154) expression **(C)** and decreased NEMO expression in $CD3^+$ cells and $CD14^+$ cells **(D)**. Sequencing of the *IKBKG* gene revealed hemizygous mutation c.262_264delGAG in exon 3 (red arrow) **(E)**. NEMO, nuclear factor kappa beta essential modulator.

decreased sweating indicated by starch-iodine test (12) (**Figure 1B**). Laboratory findings showed an elevated white blood cell count (25,000/μL with 38% polymorphonuclear neutrophils and 55% lymphocytes) and C-reactive protein concentration (7.5 mg/dL). His serum immunoglobulin levels were as follows: IgG 87 mg/dL (reference range: 300-700 mg/dL), IgG1 41.5 mg/dL (136.9-497.8 mg/dL), IgG2 22.4 mg/dL (42.3-159.6 mg/dL), IgA 10 mg/dL (9-55 mg/dL), IgM 148 mg/dL (51-188 mg/dL), and IgE <5.0 IU/mL (≤20 IU/mL). Furthermore, specific antibodies for hepatitis B virus and pertussis-toxin were not detectable despite previous vaccination. His complement components were normal. B lymphocytes were present in peripheral blood ($CD19^+$ cells: 17%) (**Supplementary Figure 1**). Chest X-ray findings were normal for the lung and left ribs. Computed tomography of the chest showed that the mass was approximately 2.7 cm in diameter, with poor internal contrast, and that it extended contiguously from the focal osteolytic lesion of the left seventh rib. We started treatment with cefazolin and immunoglobulin replacement for hypogammaglobulinemia. Growth of Gram-positive cocci in chains from three sets of blood culture led to a change in antibiotic from cefazolin to ceftriaxone. His fever resolved and penicillin-susceptible *S. pneumoniae* serotype 6C was isolated. We therefore de-escalated to ampicillin and started trimethoprim-sulfamethoxazole for prophylaxis on the assumption of a diagnosis of hyper IgM syndrome, because hyper IgM syndrome is associated with *Pneumocystis* pneumonia (13). However, the size of the mass did not decrease, and debridement of the abscess and bone curettage were necessary to treat the lesion. Thereafter, the size of the abscess did not increase. Intravenous ampicillin was switched to oral amoxicillin after inflammatory signs improved, and he was discharged on hospital day 51. He completed a 6-month course of antibiotic treatment for chronic rib osteomyelitis with maintenance of IgG trough levels over 700 mg/dL without relapse of the lesion. At 16 months of age, 2 maxillary central conical incisors have erupted.

On the basis of his family history of X-linked recessive form of inheritance and phenotype, X-linked recessive hyper IgM syndrome was suspected. There are two types of X-linked recessive hyper IgM syndrome. Type 1 is the most frequent and is associated with CD40 ligand abnormality. The other type is XL-EDA-ID associated with NEMO abnormality. Flow cytometric analysis of peripheral blood revealed normal expression of CD40 ligand (**Figure 1C**) and decreased NEMO expression in $CD3^+$ cells and $CD14^+$ cells (**Figure 1D**). Analysis of the *IKBKG* gene by long-range PCR and the Sanger sequence (14) revealed a novel in-frame deletion of a codon (c.262_264delGAG), which resulted in one amino acid microdeletion (ΔE88) in exon 3 (**Figure 1E**). The primer pairs and methods for this analysis were shown in **Supplementary Table**.

We further examined his mother and conducted a functional analysis of the variant *in vitro*. Flow cytometric analysis of maternal peripheral blood showed mixed expression of normal and mutated NEMO (**Figure 2A**). The same heterozygous variant (c.262_264delGAG) was also found in the mother (**Figure 2B**). She was ultimately diagnosed with IP on the basis of hypodontia and linear hyperpigmentation that followed the Blaschko line on her left arm (**Figure 2C**). She had no history of suspected immunodeficiency. An NF-κB reporter gene analysis measuring the activity of *IKBKG* variants using NEMO-deficient HEK293 cells (14) showed loss of activity of the *IKBKG* variant ΔE88, as compared with the wild type, and no response against tumor necrosis factor-alpha (TNF-α) (**Figure 3**). Ultimately, we diagnosed XL-EDA-ID due to *IKBKG* ΔE88 in our patient.

The family history of infantile death of two maternal uncles suggested that both had XL-EDA-ID. Because their preserved umbilical cords (**Figure 4A**) were available, we extracted DNA

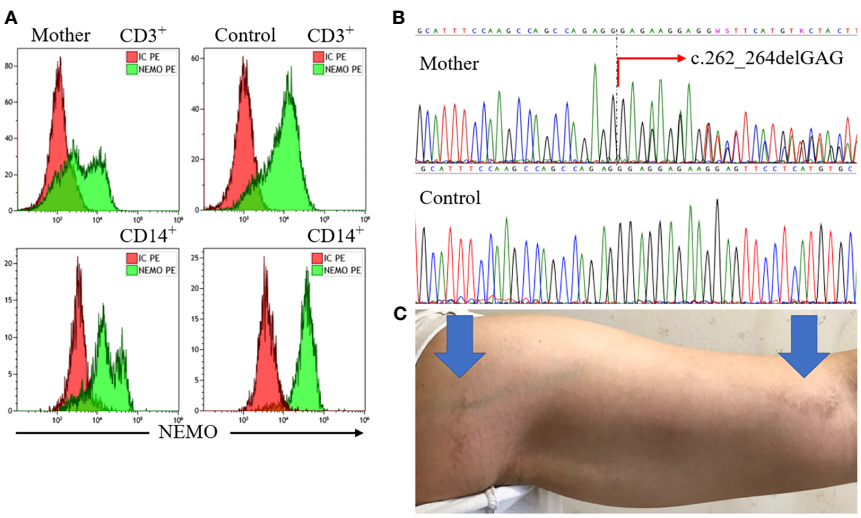

FIGURE 2 | Findings for the mother. Flow cytometric analysis of maternal peripheral blood revealed mixed expression of normal and mutated NEMO **(A)**. Sequencing of the *IKBKG* gene revealed the heterozygous variant c.262_264delGAG (red arrow) **(B)**. The mother's left arm exhibited linear hyperpigmentation following the Blaschko line (blue arrows) **(C)**. NEMO, nuclear factor kappa beta essential modulator.

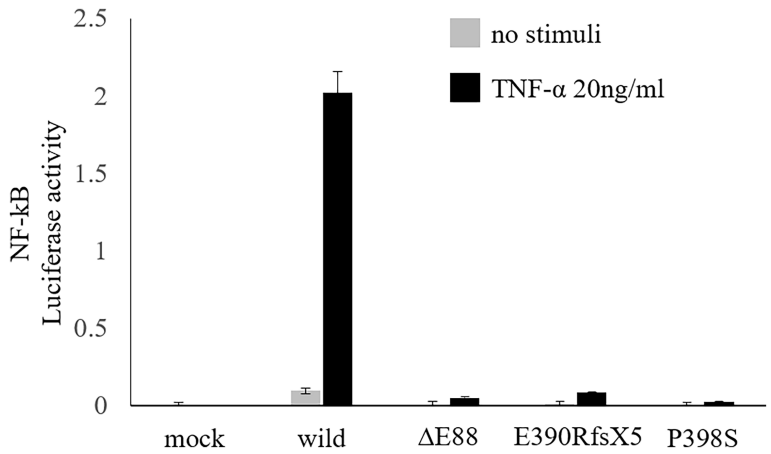

FIGURE 3 | Functional analysis of the variant. NF-κB reporter gene analysis using NEMO-deficient HEK293 cells revealed a loss of activity of the *IKBKG* variant ΔE88, as compared with the wild type, and no response against TNF-α. Hypomorphic variants already reported, i.e., E390RfsX5 and P398S (14), were also included to the analysis to ensure the accuracy of this experiment. Data are shown as the mean and standard error of triplicate measurements. NF-κB, nuclear factor kappa beta; NEMO, nuclear factor kappa beta essential modulator; TNF-α, tumor necrosis factor-alpha.

with ZR-DuetTM DNA/RNA MiniPrep Plus kit (Zymo Research), in accordance with the manufacturer's instructions, and performed sequencing with the Sanger sequencing method (**Figure 4B**). These old samples were probably DNA-fragmented by aging; thus, newly designed primers for a shorter target gene of exon 3 of *IKBKG* gene were used instead of long-range PCR (**Supplementary Table**). Although each variant was identified in a heterozygote because of the existence of a pseudogene, both samples were confirmed to be from the uncles by confirming the presence of male-specific *SRY* gene (**Supplementary Table** and **Supplementary Figure 2**). Analysis of their preserved umbilical cords enabled us to diagnose XL-EDA-ID in both maternal uncles. We further examined the maternal grandmother of the index patient. In contrast to the index patient, the mother, and two maternal uncles, sequencing of the *IKBKG* gene using the peripheral blood lymphocytes did not reveal any variants in exon 3 (**Supplementary Figure 3**).

DISCUSSION

This report used preserved umbilical cord as a tool to diagnose IEI in deceased relatives, after diagnosis of XL-EDA-ID in an infant with a novel variant. Although the maternal uncles died during infancy, the outcomes would likely be different now because of improvements in medical care during the last 40

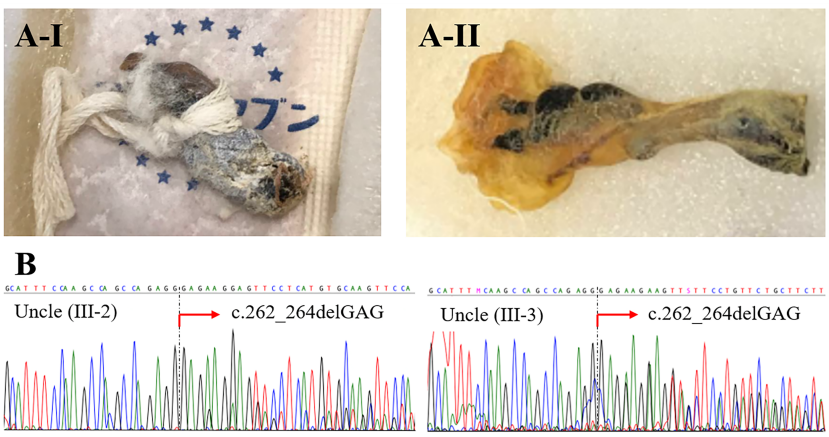

FIGURE 4 | Preserved umbilical cords from deceased relatives. A-I is from the older uncle (Uncle III-2), and A-II is from the younger uncle (Uncle III-3) **(A)**. Sequencing of the *IKBKG* gene revealed the heterozygous variant c.262_264delGAG in both uncles **(B)**.

years, particularly the availability of immunoglobulin products and effective antibiotics. Identification of the same variant in the deceased relatives confirmed the diagnosis and revealed varied phenotypes at the new gene variant site.

Previous reports have described more than 70 *IKBKG* gene variants, but not ΔE88 (15). NEMO is a regulatory protein comprising 419 amino acids and is made up of several domains such as coiled-coil motifs, leucine zipper domain, and zinc finger domain (16). A previous review of phenotypes of individuals with *IKBKG* variants assessed clinical phenotype, infectious susceptibility, and immune capacity and found that several variants have genotype-phenotype correlations (11). Our patient with the ΔE88 variant had many similarities in coiled-coil motif 1, including high susceptibility to polysaccharide encapsulated bacteria due to pneumococcal osteomyelitis and impaired response to TNF-α. However, this is the first report to link this site to hyper IgM and deaths (11, 15). Not all the effects of *IKBKG* variants have been revealed and, similarly, there is no correlation between disease severity and the site of an IP variant in females (17).

When genes of interest have pseudogenes, employing high-throughput sequencing technologies such as targeted gene panels or exome sequencing have difficulty in reliable variant identification (18). In our index patient, the variant was demonstrated by long-range PCR with the removal of the pseudogene and the Sangar sequence (14). The use of next-generation sequencing might have missed the variant.

Although the family history of X-linked recessive form of inheritance and phenotype was a key to diagnose XL-EDA-ID in the index patient, the maternal grandmother did not have the same *IKBKG* variant as the index patient, the mother, and two maternal uncles. This is possibly due to maternal germinal mosaicism, reported in other IEI such as X-linked agammaglobulinemia (19) and X-linked severe combined immunodeficiency (20).

Diagnosis of XL-EDA-ID from preserved umbilical cord tissue was challenging because PCR detection of long nucleotides was not possible, perhaps because of DNA fragmentation. The newly designed primers for shorter target genes, which were based on the index patient's variant, enabled sequencing of fragmented DNA from preserved umbilical cord. The pseudogene allele might affect the assay, resulting in identification of a gene variant in a heterozygote in males. The hemizygous state for the variant could not be confirmed in the maternal uncles because of this pitfall. Contamination of the umbilical cord by maternal blood was also a possibility; PCR confirmation of the *SRY* gene just demonstrated that the preserved umbilical cord contained male origin tissue.

The analysis of preserved umbilical cord is limited in the areas where it is available; however, the customs of preserving umbilical cord are documented among many countries and cultures outside Japan (4).

In summary, we identified a novel *IKBKG* variant that causes EDA-ID and this is the first to describe the analysis of preserved umbilical cord tissue to diagnose IEI in deceased relatives. When investigating IEI in a family, analysis of preserved umbilical cord tissue from decedents can yield information important for diagnosis of living relatives and help clarify the phenotypes of new gene variant sites.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Gifu University. Written informed consent to participate in this study was provided by the participants' parents. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Patient's management: SI, YA, CI, and AS. Manuscript preparation: SI and YA. Study concept: HK. Study Design: YA, HO, and HK. Literature search: SI, YA, and HK. Data analysis/interpretation: YA, YM, CI, HO, and HK. Manuscript editing: CI, HO, HK, and AS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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The Primary Immunodeficiency Database in Japan

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The Primary Immunodeficiency Database in Japan (PIDJ) is a registry of primary immunodeficiency diseases (PIDs) that was established in 2007. The database is a joint research project with research groups associated with the Ministry of Health, Labor and Welfare; the RIKEN Research Center for Allergy and Immunology (RCAI); and the Kazusa DNA Research Institute (KDRI). The PIDJ contains patient details, including the age, sex, clinical and laboratory findings, types of infections, genetic analysis results, and treatments administered. In addition, web-based case consultation is also provided. The PIDJ serves as a database for patients with PIDs and as a patient consultation service connecting general physicians with PID specialists and specialized hospitals. Thus, the database contributes to investigations related to disease pathogenesis and the early diagnosis and treatment of patients with PIDs. In the 9 years since the launch of PIDJ, 4,481 patients have been enrolled, of whom 64% have been subjected to genetic analysis. In 2017, the Japanese Society for Immunodeficiency and Autoinflammatory Diseases (JSIAD) was established to advance the diagnosis, treatment, and research in the field of PIDs and autoinflammatory diseases (AIDs). JSIAD promotes the analysis of the pathogenesis of PIDs and AIDs, enabling improved patient care and networking *via* the expansion of the database and construction of a biobank obtained from the PIDJ. The PIDJ was upgraded to "PIDJ ver.2" in 2019 by JSIAD. Currently, PIDJ ver.2 is used as a platform for epidemiological studies, genetic analysis, and pathogenesis evaluation for PIDs and AIDs.

Keywords: primary immunodeficiency, Primary Immunodeficiency Database in Japan, Japanese Society for Immunodeficiency and Autoinflammatory Diseases, consultation, genetic analysis, pathogenesis

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INTRODUCTION

Primary immunodeficiency diseases (PIDs) are rare and genetically heterogeneous disorders that impair the immune system. Recent studies have indicated the prevalence of >400 causative genes, and genetic analysis plays an important role in confirming the PID diagnosis and the selection of treatment options, which include the use of hematopoietic stem cell transplantations, gene therapy, and biological agents. Because PIDs are rare diseases, the compilation of patient details, including genetic analysis and clinical information, can contribute significantly toward evaluating the pathogenesis involved and the establishment of optimal treatment methods. Given this background, a registry of patients with primary immunodeficiency (PIDJ: Primary Immunodeficiency Database in Japan) was established in 2007, enabling an overview of Japanese patients with PIDs.

Before the PIDJ Project

Before the launch of the PIDJ project, Japan had a retrospective, paper-based registration system. The first nationwide survey related to PIDs in Japan was performed in 1979, which was supported by the Japan Ministry of Health, in which 497 patients were registered (1). During registration, the survey included the numbers of each type of PIDs, patient age at the time of diagnosis, patient status at the time of registration, familial incidence of PIDs, and any associated complications. However, molecular analysis and sample stocking were not performed in that survey. When the causative gene associated with each patient with PIDs was identified, it was registered in each institution's private database, as a nationwide database had not been established.

The PIDJ Project (2007~2017)

PIDs are rare diseases, with low numbers of patients but highly variable symptoms, severity, and complications, making diagnosis by non-specialists difficult. The disease can be fatal if the diagnosis is delayed or if no appropriate therapeutic intervention is performed; therefore, consultations with PID expert doctors are required. Therefore, in 2007, we launched a website for PIDJ at the RIKEN Research Center for Allergy and Immunology (RCAI) to facilitate consultations for PID patients with local physicians or expert doctors from 13 universities across Japan. In the PIDJ network, general physicians evaluating potential PID patients consult expert doctors with experience in PID diagnosis and register for PIDJ with informed consent from the patients. The clinical information of the patients is added *via* the internet, and PID experts advise general physicians consulting with the patient. Where further analyses are required, patients' samples are sent to RCAI or PID expert doctors to perform immunological analysis, including FACS and genetic and functional evaluations, and patient samples are preserved for future use. Genetic analysis is performed at the Kazusa DNA Research Institute (KDRI). The important goals are to enable accurate diagnosis, recommend appropriate treatments, and provide support to connect PID patients to specialized medical institutions for prompt and appropriate medical care. Thus, consultation, registration, sample stocking, molecular diagnosis, functional analysis, and advice from PID expert doctors can easily be achieved through the PIDJ network.

In the 9 years (from 2007 to 2017) since the launch of PIDJ, 4,481 patients have been registered. Genetic analysis has been performed in 2,869 patients (64% of all registered patients), and the causative gene was identified in 804 cases. The most common diagnosis was autoinflammatory disorders (39%), followed by predominantly antibody deficiencies (14.4%), combined immunodeficiencies with syndromic features (8.7%), diseases of immune dysregulation (8.7%), congenital defects of phagocytes (7.9%), and combined immunodeficiencies (3.9%) (**Table 1**).

Moreover, PIDJ has contributed to the identification of novel causative genes associated with PIDs. For patients in whom no mutations in known PID-causing genes are detected, cases with similar clinical symptoms and laboratory findings are selected for detailed analysis to identify novel causative genes. We have identified several causative genes of PIDs by using PIDJ database (2–13).

PIDJ ver.2, Now and Beyond

In 2017, the Japanese Society for Immunodeficiency and Autoinflammatory Diseases (JSIAD) was established to advance the diagnosis, treatment, and research in the field of PIDs and autoinflammatory diseases (AIDs). The PIDJ was upgraded to "PIDJ ver.2" in 2019 by JSIAD. With the expansion of the database and construction of a biobank, PIDJ ver.2 is being used as a platform for epidemiological studies, genetic analysis, and pathogenesis evaluation for PIDs and AIDs. Consultation, registration, sample stocking, molecular diagnosis, functional analysis, and advice from PID expert doctors are being provided using PIDJ ver.2. The PIDJ committees are organized in JSIAD to respond to consultations from general physicians. Genetic analysis (by NGS) and sample stocking are performed at the KDRI. The JSIAD collaborating facilities (81 facilities as of June 2020) participate in PIDJ ver.2 as joint research facilities and play a role in the diagnosis and treatment of PIDs and AIDs, as well as research and education about the topic. Further, on the basis of the IUIS classification (14, 15), JSIAD has developed a panel of genes recommended for genetic testing for each PID and AID related to clinical usefulness and validity (**Table 2**).

DISCUSSION

PIDs include over 400 diseases caused by mutations in single genes, which makes them difficult to diagnose by general

TABLE 1-1 | The total numbers of registered patients (2008~2016).

IUIS classification	Number of Patients	(%)
Combined immunodeficiencies	170	(3.9%)
Combined immunodeficiencies with syndromic features	377	(8.7%)
Predominantly antibody deficiencies	624	(14.4%)
Diseases of immune dysregulation	375	(8.7%)
Congenital defects of phagocytes	343	(7.9%)
Defects in intrinsic and innate immunity	141	(3.2%)
Autoinflammatory disorders	1692	(39%)
Complement deficiencies	24	(5.5%)
Phenocopies of PID	410	(9.5%)
Others	151	(3.5%)
Total	4307	

TABLE 1-2 | The total numbers of genetically diagnosed patients (2008~2016).

IUIS Classification	Genetic Defect	Number of Patients	(%)
Combined immunodeficiencies	*IL2RG*	51	(6.3%)
	CD40L	36	(4.4%)
	RAG1	13	(1.6%)
Combined immunodeficiencies with syndromic features	*WAS*	82	(10.1%)
	STAT3	31	(3.8%)
	ATM	28	(3.4%)
	IKBKG	16	(1.9%)
Predominantly antibody deficiencies	*BTK*	69	(8.5%)
Diseases of immune dysregulation	*XIAP*	15	(1.8%)
	UNC13D	15	(1.8%)
	CTLA4	14	(1.7%)
	PRF1	13	(1.6%)
Congenital defects of phagocytes	*CYBB*	54	(6.7%)
	NCF2	20	(2.4%)
	ELANE	11	(1.3%)
Defects in intrinsic and innate immunity	*STAT1*	12	(1.4%)
Autoinflammatory disorders	*MEFV*	104	(12.9%)
	NLRP3	15	(1.8%)
	TNFAIP3	13	(1.6%)
Total	89 genes	804	

The causative genes identified in more than 10 patients are listed in the Table.

TABLE 2 | Target genes responsible for PIDs and AIDs.

IUIS Classification (2019)	Name of Panel Set	Target Genes
Table 1	Combined Immunodeficiency (1)	IL2RG, JAK3, IL7R, RAG1, RAG2, DCLRE1C, ADA, PNP, ZAP70, LIG4, NHEJ1, TBX1
	Combined Immunodeficiency (2)	AK2, CORO1A, FOXN1, PRKDC, PTPRC, STAT5B, ORAI1, STIM1, MAGT1, RAC2, CHD7, SEMA3E, POLE, ATM, CD3D,CD3E, CD247, LAT
	MHC deficiency	TAP1, TAP2, B2M, CIITA, RFXANK, RFX5, RFXAP
Table 2	Wiskott–Aldrich syndrome	WAS, ARPC1B, CDC42, WIPF1
	Hyper IgE syndromes	STAT3, TYK2, IL6R, ZNF341, ERBIN, TGFBR1,TGFBR2, SPINK5,PGM3, CARD11, DOCK8
	Immuno-osseous dysplasias	SMARCAL1, RNU4ATAC, EXTL3
	DNA mismatch-repair deficiency	ATM, MRE11, NBN, RAD50, LIG4, NHEJ1, DCLRE1C, PRKDC, DNMT3B, ZBTB24, CDCA7, HELLS, RNF168, MCM4, BLM
	Anhidrotic ectodermodysplasia with immunodeficiency	IKBKG, NFKBIA, IKBKB, ORAI1
Table 3	Profoundly decreased or absent B cells	BTK, IGHM, IGLL1, CD79A, BLNK, PIK3CD, PIK3R1, TCF3, SLC39A7, TRNT1, IKZF1, IKZF3
	Hyper-IgM syndromes	CD40LG, AICDA, CD40, UNG, INO80, PIK3CD, PIK3R1, PTEN, IKBKG
	Common variable immunodeficiency (CVID) (1)	TNFSF12, TNFSF13, TNFRSF13B, TNFRSF13C, CD19, CR2, PLCG2, IKZF1, IKZF3, NFKB1, NFKB2, SEC61A1, IRF2BP2, ATP6AP1, ARHGEF1, SH3KBP1, DNMT3B, ZBTB24, CDCA7, HELLS
	Common variable immunodeficiency (CVID) (2)	ICOS, PLCG2, LRBA, CTLA4, IL21R, MALT1, MSN, CARD11,BCL10, ITK, PIK3CD, PIK3R1, NFKB1, NFKB2
Table 4	Familial hemophagocytic lymphohistiocytosis (FHL syndromes)	PRF1, UNC13D, STX11, STXBP2, FAAP24, SLC7A7, LYST,RAB27A, AP3B1, AP3D1, SH2D1A, XIAP
	Autoimmune lymphoproliferative syndrome	FAS, FASLG, CASP8, CASP10, NRAS, KRAS, AIRE, FOXP3, IL2RA, CTLA4, LRBA, STAT3, SH2D1A, IKZF1, PIK3CD, PIK3R1, PRKCD, TNFAIP3
	IPEX syndromes	FOXP3, IL2RA, IL2RB, CTLA4, LRBA, STAT3, FERMT1, STAT1, STAT5B
	Immune dysregulation with colitis	IL10, IL10RA, IL10RB, NFAT5, TGFB1, RIPK1, FOXP3, IL2RA, CTLA4, LRBA, WAS, XIAP, CYBA, CYBB, NCF2, NCF4, TNFAIP3
	Susceptibility to EBV and lymphoproliferative conditions	SH2D1A, XIAP, CD27, RASGRP1, CARMIL2, MAGT1, PRKCD, STK4, ITK, ZAP70, MCM4, PIK3CD, PIK3R1, NFKB1, CTLA4, PRF1, STXBP2, FAS
Table 5	Congenital neutropenias (1)	ELANE, HAX1, WAS, CSF3R, SRP54, CXCR4
	Congenital neutropenias (2)	GFI1, G6PC3, SLC37A4, TAZ, VPS13B, USB1, JAGN1, CLPB
	Shwachman–Diamond syndrome	SBDS
	Leukocyte adhesion deficiency	ITGB2, SLC35C1, FERMT3, RASGRP2

(Continued)

TABLE 2 | Continued

IUIS Classification (2019)	Name of Panel Set	Target Genes
	Chronic granulomatous disease (CGD)	CYBB, CYBA, NCF2, NCF4, G6PD
	Congenital defects of phagocyte	RAC2, ACTB, FPR1, CTSC, WDR1, MRTFA, SLC11A1, CEBPE, G6PD, MPO
	Familial defects of dendritic cells	GATA2, CSF2RA, CSF2RB, IRF7, IRF8
IUIS classification (2019)	Name of panel set	Target genes
Table 6	Mendelian susceptibility to mycobacterial disease (MSMD)	IL12RB1, IL12B, IL12RB2, IL23R, IFNGRl, IFNGR2, STAT1,CYBB, IRF8, TYK2, RORC, JAK1, IKBKG, GATA2
	TLR signaling pathway deficiency with bacterial susceptibility	IRAK4, MYD88, TIRAP, IKBKG, NFKBIA, IKBKB, RPSA, NKX2-5, RBCK1
	Chronic mucocutaneous candidiasis; CMC	IL17RA, IL17F, STAT1, TRAF3IP2, RORC, AIRE, STAT3, IL12RB1, IL12B, CARD9
	Predisposition to severe viral infection	STAT1, STAT2, IRF7, IFNAR1, FCGR3A, IFIH1, TLR3, TBK1,DBR1, IRF8, MCM4, TMC6, TMC8, CXCR4
Table 7	Autoinflammatory disorders	ADA2, NLRC4, TNFAIP3
	Aicardi–Goutieres syndrome (AGS)	TREX1, RNASEH2B, RNASEH2C, RNASEH2A, SAMHD1, ADAR, IFIH1
	Cryopyrin-associated periodic fever syndrome	NLRP3
	Hyper-IgD syndrome	MVK
	Pyogenic sterile arthritis, pyoderma gangrenosum, acne (PAPA) syndrome	PSTPIP1
	TNF receptor-associated periodic syndrome (TRAPS)	TNFRSF1A
	Nakajo–Nishimura syndrome	PSMB8
	Familial Mediterranean fever	MEFV
	Blau syndrome	NOD2
Table 8	Complement deficiencies	C1QA, C1QB, C1QC, C1R, C1S, C2, C3, C5, C6, C7, C8A, C8B, C9, CFB, CFI, CFP, MASP2, MBL2
	Complement deficiencies (hereditary angioedema)	SERPING1, F12, ANGPT1, PLG, CD55, CD59
Table 9	Dyskeratosis congenita	DKC1, TERC, TERT, TINF2, RTEL1, ACD, WRAP53, PARN, CTC1, DCLRE1C

physicians. Because PIDs are rare diseases, patient registration is important for the diagnosis, genetic and functional analysis, and improved patient care. PIDJ was initially associated with 13 medical colleges, the RCAI, and KDRI, back in 2007. PIDJ is a nationwide network that includes patient consultations, registration, sample stocking, genetic diagnosis, molecular analysis, and advice by PID expert doctors.

A total of 4,481 patients have been registered in PIDJ, and genetic analysis has been performed in 2,869 patients (64% of all registered patients), and the causative gene was identified in 804 cases. Comparing our results with reports from registries in other countries (e.g., Europe, the United States, the Middle East, and Asia), PIDJ is characterized by a large number of registered AID patients (especially with *MEFV* mutations) (16–28). On the other hand, with regard to PIDs, adult cases that had already been diagnosed and cases that have been followed only with γ-globulin administration may not have been registered in PIDJ. This is the limitation of this registration, and future efforts should be made to ensure that all PID patients in Japan will be registered in PIDJ. We are conducting a survey of all PIDs patients in Japan to identify missing cases in the registration and are working on registering all the PIDs patients for PIDJ (29, 30).

PIDJ was upgraded to PIDJ ver.2 with the establishment of JSIAD in 2017 and has been expanded further, with additional functions to date. The PIDJ network will help doctors and researchers perform various analyses, improve disease prognosis, and advance understanding of the human immune system.

AUTHOR CONTRIBUTIONS

KM-S did the conception and design. KM-S, KI, and AE analyzed the data. KM-S and KI wrote the manuscript. SN, YS, and KI provided critical discussion, supervised the study, and edited the manuscript. All authors reviewed the paper. All authors contributed to the article and approved the submitted version.

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Clinical Courses of IKAROS and CTLA4 Deficiencies: A Systematic Literature Review and Retrospective Longitudinal Study

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IKAROS and CTLA4 deficiencies are inborn errors of immunity and show similar clinical phenotypes, including hypogammaglobulinemia and autoimmune diseases (ADs). However, the differences in clinical features and pathogenesis of these are not fully understood. Therefore, we performed systematic literature reviews for IKAROS and CTLA4 deficiencies. The reviews suggested that patients with IKAROS deficiency develop AD earlier than hypogammaglobulinemia. However, no study assessed the detailed changes in clinical manifestations over time; this was likely due to the cross-sectional nature of the studies. Therefore, we conducted a retrospective longitudinal study on IKAROS and CTLA4 deficiencies in our cohort to evaluate the clinical course over time. In patients with IKAROS deficiency, AD and hypogammaglobulinemia often develop in that order, and AD often resolves before the onset of hypogammaglobulinemia; these

observations were not found in patients with CTLA4 deficiency. Understanding this difference in the clinical course helps in the clinical management of both. Furthermore, our results suggest B- and T-cell-mediated ADs in patients with IKAROS and CTLA4 deficiencies, respectively.

Keywords: IKAROS deficiency, CTLA4 deficiency, systematic literature review, retrospective longitudinal study, clinical course

INTRODUCTION

Common variable immunodeficiency (CVID) is the most prevalent disease of inborn errors of immunity (IEI) and is characterized by hypogammaglobulinemia with poor specific antibody production. In addition to increased susceptibility to recurrent bacterial infections, clinical manifestations of CVID include noninfectious complications, such as autoimmune diseases (ADs), enteropathy, lymphoproliferation/granulomatous diseases, and malignancy (1). Although CVID is clinically and genetically heterogeneous, identifying underlying genetic defects with the help of latest advances in sequencing technology can help classify CVID into homogeneous subgroups. Indeed, several next-generation sequencing studies have identified that 15%-30% of patients with CVID have candidate variants in genes including *NFKB1*, *NFKB2*, *CTLA4*, *LRBA*, *IKZF1*, *STAT3*, and *PIK3CD* (2).

Identifying genetic defects can help understand the pathogenesis of hypogammaglobulinemia or noninfectious complications. For example, IKAROS encoded by *IKZF1* is a key transcription factor of hematopoietic development, particularly early B-cell development. Patients with IKAROS haploinsufficiency (HI) or haploinsufficiency of the dimerization domain (DD) present with progressive B-cell deficiency, whereas IKAROS dominant-negative (DN) results in more severe manifestations involving B-, T-, and myeloid cell defects (collectively termed as IKAROS deficiency) (3–6). IKAROS also plays a role in tumor suppression. Somatic variants have been recurrently observed in B-cell precursor acute lymphoblastic leukemia (BCP-ALL), and patients with germline variants can develop BCP-ALL (3). Additionally, CTLA4 is an essential negative regulator of T-cell immune responses, and is expressed on regulatory (Treg) and activated effector T cells. CTLA4 HI (termed as CTLA4 deficiency) results in a hyperactivated immune system with T-cell infiltration in organs, autoimmunity, or both (7, 8). Although not completely clear, bone marrow infiltration of T cells or impaired germinal center formation may lead to hypogammaglobulinemia with reduced number of memory B cells (9). Considering this condition, the clinical use of abatacept, a CTLA4 fusion protein, is a reasonable therapeutic option.

Subdividing CVID by identifying its genetic defects has reduced the sample size of the studies. Early studies on CVID, especially cross-sectional studies, that clarified the clinical manifestations and appropriate management practices used large heterogeneous cohorts of 100 to >1,000 patients (10). However, recent studies on a monogenic disease included smaller cohorts of 10-100 patients (3, 4). Although studies on homogeneous cohorts could more accurately characterize the clinical features of the diseases, errors and biases may occur due to the small size. Therefore, world surveys or systematic literature reviews have been conducted to increase the sample size (11, 12). However, such studies are associated with potential problems as described below, thus warranting careful evaluations. Moreover, longitudinal studies are more suitable for studying a small number of patients rather than a large cohort due to the efficiency of time and cost (**Supplemental Table 1**) (13).

The primary aim of this study was to clarify the limitations of performing a literature review on IKAROS and CTLA4 deficiencies and to emphasize that a longitudinal study could compensate for its limitations. The secondary aim was to provide a possible pathogenesis for complications through a longitudinal study. This study focused on IKAROS and CTLA4 deficiencies from among the many known genetic defects because cohorts for these conditions have been previously described (4, 11) and the pathogenesis of at least one complication is well understood. Here, we performed systematic literature reviews for IKAROS and CTLA4 deficiencies and highlighted the associated problems, including case-publication bias, lack of data, and lack of information about the clinical course over time. This is most likely due to the presence of multicenter and cross-sectional studies. Furthermore, we conducted a retrospective longitudinal study on IKAROS and CTLA4 deficiencies in our Japanese cohort and determined a different onset order of hypogammaglobulinemia and AD in the two conditions. Therefore, this study aimed to emphasize that longitudinal studies can help understand the clinical presentation of diseases, such as IEI, with a small sample size and may provide a further understanding of the pathogenesis and aid in better clinical management.

MATERIALS AND METHODS

Systematic Literature Review

We searched PubMed and Web of Science databases for articles published in English from April 2012 to June 2021 on IKAROS deficiency and from September 2014 to June 2021 on CTLA4 deficiency. The search terms were a "IKAROS haploinsufficiency", "IKAROS haploinsufficient", "IKAROS deficiency", "IKAROS deficient", "IKAROS mutation", "IKAROS mutations", "IKAROS variant", "IKAROS variants", "IKZF1 mutation", "IKZF1 mutations", "IKZF1 variant", and "IKZF1 variants" for IKAROS deficiency, and "cytotoxic T-lymphocyte-associated protein 4

haploinsufficiency", "cytotoxic T-lymphocyte-associated protein 4 haploinsufficient", "cytotoxic T-lymphocyte-associated protein 4 deficiency", "cytotoxic T-lymphocyte-associated protein 4 deficient", "cytotoxic T-lymphocyte-associated protein 4 insufficiency", "cytotoxic T-lymphocyte-associated protein 4 insufficient", "CTLA4 haploinsufficiency", "CTLA4 haploinsufficient", "CTLA4 deficiency", "CTLA4 deficient", "CTLA4 insufficiency", "CTLA4 insufficient", "CTLA-4 haploinsufficiency", "CTLA-4 haploinsufficient", "CTLA-4 deficiency", "CTLA-4 deficient", "CTLA-4 insufficiency", "CTLA-4 insufficient", "CTLA4 mutation", "CTLA4 mutations", "CTLA4 variant", and "CTLA4 variants" for CTLA4 deficiency. A search was performed using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines for article identification, screening, eligibility, and inclusion (**Supplemental Figure 1**). Patients with insufficient details on clinical manifestations and with not functionally tested variants were excluded. However, those with nonsense or frameshift variants were included even if untested. IKAROS DN was evaluated separately as such patients have distinct clinical features.

Retrospective Longitudinal Study

We identified 16 patients with IKAROS deficiency and 31 with CTLA4 deficiency in our registry of IEI referred to the Tokyo Medical and Dental University. One patient with IKAROS DN was excluded. Data on clinical presentation with its course over time, serum immunoglobulin levels, and treatment were retrospectively surveyed by reviewing medical records or questionnaires sent to physicians. Patients 1.1-20.1 and 22.1 have been previously reported (4, 5, 9, 11, 14–20). AD onset was calculated as the number of AD onset per 1 person-years, which is the sum of the patient follow-up duration (years). Incidence of AD remission was calculated as the number of AD remission per 1 person-years, which is the sum of the patient AD duration (years).

Data Evaluation

Enteropathy, granulomatous and lymphocytic interstitial lung disease (GLILD), central nervous system (CNS) involvement, and other symptomatic lymphoproliferation/granulomatous diseases were included in AD as they are inflammatory complications and most likely associated with impaired immunological tolerance and lymphocyte infiltration (11). Asymptomatic lymphoproliferation, including lymphadenopathy and splenomegaly, was excluded from AD. The onset of hypogammaglobulinemia was defined as the onset of susceptibility to infection if serum immunoglobulin levels were not tested. Additionally, IgA and specific antibody deficiencies were excluded from hypogammaglobulinemia to emphasize the role of severe B-cell development/differentiation arrest.

In the longitudinal study, AD remission was defined as absent or minimal symptoms, normal laboratory data without treatment, and no relapse until the last follow-up. For patients who underwent hematopoietic stem cell transplantation (HSCT), onset and remission were evaluated for the period before HSCT. Furthermore, if hypogammaglobulinemia and AD simultaneously identified, AD onset was considered to be before that of hypogammaglobulinemia, because serum IgG levels and B-cell counts were further decreased in all available patients.

Statistical Analyses

For statistical analyses and construction of Kaplan-Meier curves, GraphPad Prism 8 software (GraphPad) was used. The groups were compared using Mann-Whitney *U*-test or Wilcoxon signed-rank test for numerical data and chi-square test for non-numerical data. Moreover, 95% confidence intervals (CIs) were calculated using the exact binomial method (Clopper-Pearson method). Differences were considered significant at P values of <0.05. For constructing Kaplan-Meier curves, we defined censoring as loss of follow-up or HSCT.

Functional Assay

Electrophoresis mobility shift assay (EMSA) and co-immunoprecipitation (Co-IP) assay were performed as previously described (4). For EMSA, HEK293T cells were transfected with pCMV3-HA-IKAROS (wild-type [WT] or mutants). Then, the nuclear protein was extracted and incubated with DY682 infrared dye-labeled double-strand IK-bs4 probe (forward: 5'-TGACAGGGAATACACATTCCC AAAAGC-3'; reverse: 5'-GCTTTTGGGAATGTGTATTCCCTG TCA-3'). DNA-protein complexes were separated using acrylamide gels and analyzed using an Odyssey CLx infrared scanner (Li-Cor). For Co-IP, HEK293T cells were co-transfected with pFLAG-CMV2-IKAROS (WT) and pCMV3-HA-IKAROS (WT or mutants). Then, cell lysates were immunoprecipitated with Protein G-Sepharose 4 Fast Flow (GE Healthcare) and mouse anti-FLAG antibody (F3165, Sigma), eluted, and separated using standard sodium dodecyl sulftate-polyacrylamide gel electrophoresis (SDS-PAGE). Finally, western blotting was performed using the rabbit anti-HA antibody (H6908, Sigma).

Intracellular CTLA4 expression was evaluated using flow cytometry. Peripheral blood mononuclear cells stimulated with anti-CD3/CD28 beads were stained with anti-CD4-PC7 (SFCI12T4D11 (T4), Beckman Coulter), fixed, permeabilized, and stained for anti-CTLA4-PE-Cy5 (BNI3, BD Biosciences) and anti-FOXP3-Alexa Fluor 647 (236A/E7, eBioscience).

RESULTS

Systematic Literature Reviews

First, systematic literature reviews of IKAROS and CTLA4 deficiencies were performed, and 122 and 177 articles, respectively, were identified. Of those, 19 and 28 articles were considered eligible for systematic review. Furthermore, 95 and 246 patients were reported in these articles, and, after excluding patients to both groups, 90 and 179 unique patients remained for data analysis, respectively (**Supplemental Figure 1** and **Supplemental Table 2**). Previous reviews and world surveys have described genetic and immunological analyses, complication types, treatment, and outcomes (9, 11, 12, 21). Therefore, we primarily focused on complications and their clinical course.

IKAROS Deficiency

This systematic review evaluated 90 patients with IKAROS deficiency bearing 28 distinct *IKZF1* variants: 66 patients (20 variants) with IKAROS HI, including large deletion and early truncation; 16 patients (6 variants) with IKAROS DD; and 8 patients (two variants) with IKAROS DN (**Table 1**). Data for IKAROS HI and DD were combined and evaluated as the number of patients was small and a similar tendency was observed in both groups (**Table 1**, **Supplemental Figure 2** and **Supplemental Table 3**). However, patients with IKAROS DN were separately evaluated as they had a phenotype distinct from CVID. In patients with IKAROS HI and DD, the median age and interquartile range at the last follow-up were 24 (13-45) years. Of 82 patients, 55 (67.1%) were symptomatic, and the median age at onset was 9 (4-19) years. Kaplan-Meier curves were constructed for 73, 72, and 7 5patients, including asymptomatic patients, whose ages at the onset of hypogammaglobulinemia, AD, and malignancy were available (**Figure 1A**). The cumulative incidence was 44.5%, 26.1%, and 9.7% at 20 years, and 61.3%, 31.1%, and 9.7% at 40 years, respectively. Although hypogammaglobulinemia developed even after 50 years of age, the cumulative incidence curves of AD and malignancy almost peaked within the first 20 years of life. These observations differ from previous reports on increased noninfectious complications with age in patients with CVID (22). Of the 23 patients with AD, 18 (78.3%) had only one AD (**Figure 2A**). Autoimmune cytopenia and systemic AD, such as systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS), were the most common (**Figure 2B**). BCP-ALL was the most common malignancy (**Figure 2C**). When each patient was separately evaluated, AD developed earlier or simultaneously as hypogammaglobulinemia (**Figure 2D**). Indeed, of 12 ADs in 10 patients available, only three showed hypogammaglobulinemia at the onset and none had agammaglobulinemia (**Figure 2E**). Furthermore, autoantibodies were detected in many patients (**Supplemental Table 4**).

In patients with IKAROS DN, the median ages at the last follow-up and onset were 10 (3-19) and 0.6 (0.3-1) years, respectively. All developed infections, including Pneumocystis pneumonia, in infancy. One patient developed T-cell ALL at the age of 13 years, and AD was not observed.

CTLA4 Deficiency

We also evaluated 179 patients with CTLA4 deficiency bearing 61 distinct *CTLA4* variants (**Table 1**). The median age at the last follow-up was 26 (18-46) years. At onset, the median age in 133 (74.3%) symptomatic patients was 11 (6-18) years. The cumulative incidence of any manifestations was 60.2% at 20 years old and 72.4% at 40 years old, whereas that of malignancy was 3.4% at 20 years old and 12.3% at 40 years old (**Figure 1B**). However, because the ages at hypogammaglobulinemia and AD onset were unavailable in 25 (14.0%) and 38 (21.2%) patients, respectively, the cumulative incidence of each complication could not be evaluated. In available patients, hypogammaglobulinemia and malignancy developed at a significantly higher age than those with IKAROS deficiency (**Table 1** and **Supplemental Figure 3**). In contrast, 93/128 (72.7%) patients had two or more ADs without the tendency to develop earlier than hypogammaglobulinemia (**Figures 2A, D**). Although various organ-specific ADs were observed, systemic AD was rare (**Figure 2B**). Furthermore, lymphoma and gastric adenocarcinoma were the most common malignancy (**Figure 2C**).

Limitations of Systematic Literature Reviews

A major limitation of these systematic literature reviews is potential case-publication bias. For example, only two articles on IKAROS deficiency and three on CTLA4 deficiency (some patients were overlapped) described the clinical manifestations of substantial cohorts of >10 patients (3, 4, 8, 11, 23). The penetrance was 72.4%-72.7% for IKAROS HI and 67.7%-86.7% for CTLA4 deficiency in these cohorts. However, except for those five articles, the total patients showed a penetrance of 80.8% and 93.5%, respectively, suggesting an enrichment of symptomatic and likely severe or atypical cases when reported as single, few patients or family case reports (**Figure 3A**). Furthermore, in addition to the lack of clinical or laboratory data, there was little information about the clinical course over time, including the remission of complications, another major limitation due to the multicenter and cross-sectional nature of the studies (**Figure 3B**).

RETROSPECTIVE LONGITUDINAL STUDY INVOLVING OUR COHORT

These systematic literature reviews suggest that patients with IKAROS deficiency develop in the order of AD and hypogammaglobulinemia, whereas those with CTLA4 deficiency

TABLE 1 | Baseline description of individuals with IKAROS deficiency (HI and DD) and CTLA4 deficiency in the systematic literature reviews.

	IKAROS HI and DD	CTLA4 def.	*P*-value
Number of patients	82	179	
Sex (M/F)	42/40 (n = 82)	91/88 (n = 179)	0.954
Age at last follow-up	24 [13-45] (n = 76)	26 [18-46] (n = 167)	0.108
Age at onset	9 [4-19] (n = 55)	11 [6-18] (n = 119)	0.203
Age at onset of hypo-γ	10 [6-19] (n = 39)	17 [12-25] (n = 33)	**0.010**
Age at onset of AD	9 [3-14] (n = 20)	10 [6-17] (n = 101)	0.301
Age at onset of malignancy	4 [3-6] (n = 6)	33 [21-50] (n = 23)	**<0.001**

The median ages are shown [with 25th and 75th percentiles] (year).
Bold numbers indicate the statistically significant correlations.
AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

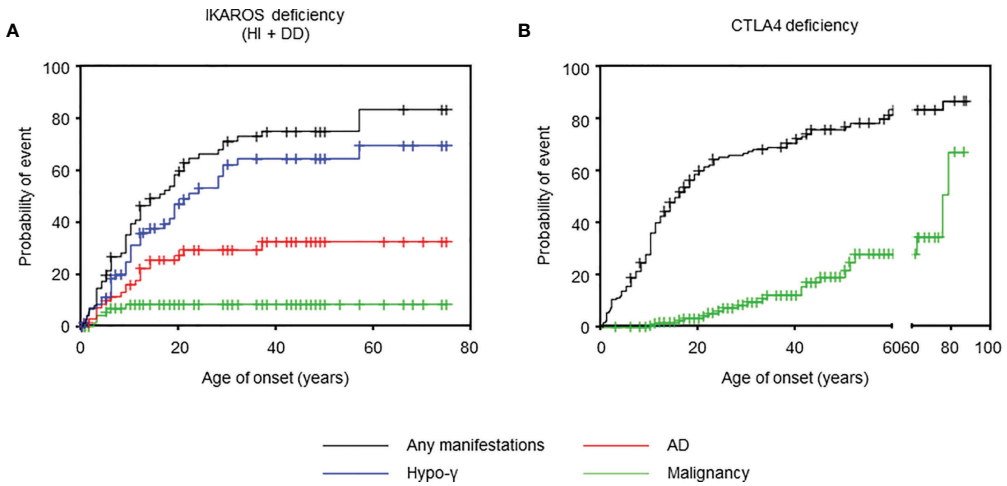

FIGURE 1 | Age of onset in IKAROS and CTLA4 deficiencies. **(A)** Cumulative incidence of any manifestations (n = 77), hypogammaglobulinemia (n = 73), autoimmune disease (n = 72), and malignancy (n = 75) in IKAROS HI and IKAROS DD. **(B)** Cumulative incidence of any manifestation (n = 158) and malignancy (n = 166) in CTLA4 deficiency. AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

do not. Therefore, to evaluate the clinical course over time in detail, we conducted a retrospective longitudinal study on IKAROS and CTLA4 deficiencies in our Japanese cohort.

Patient Cohort and Functional Assays

Our cohort identified 16 patients with *IKZF1* variants bearing 10 distinct variants and 31 patients with *CTLA4* variants bearing 16 distinct variants (**Supplemental Table 5**). First, we functionally tested three previously untested *IKZF1* variants, K157del, F490del, and H508W and three novel CTLA4 variants, V84A, C129R, and P162fs. Three *IKZF1* variants were located on the DNA-binding or dimerization domain and are predicted to affect the maintenance of zinc finger structure or the coordination of zinc atom (**Supplemental Figure 4A**). The consequences of *IKZF1* variants were examined with mutant proteins transiently expressed in HEK293T cells. Furthermore, EMSA showed that the K157del mutant protein did not bind the IKAROS consensus sequence without a DN effect, indicating a HI variant (**Supplemental Figure 4B**). Co-IP assay showed that mutants F490del and H508W failed to bind to WT IKAROS, indicating DD variants (**Supplemental Figure 4C**). Additionally, CTLA4 protein expression was measured in $FOXP3^+$ T cells from patients with *CTLA4* V84A, C129R, and P162fs variants. All three variants resulted in reduced CTLA4 expression (**Supplemental Figure 4D**). As IKAROS DN shows distinct clinical features, 15 patients with IKAROS deficiency (13 with HI and two with DD), excluding one with IKAROS DN, and 31 with CTLA4 deficiency were evaluated.

Case Reports

Herein, we describe four patients who had typical clinical courses (**Figure 4A**). Patient 1.1 with IKAROS HI is a 13-year-old boy presenting with immune thrombocytopenia (ITP) at the age of 3 years with increased platelet-associated IgG. Low serum IgA and IgM levels were observed at ITP diagnosis (IgG, 784 mg/dL; IgA, 2 mg/dL; and IgM, 9 mg/dL). His platelet counts were between 50,000 and 100,000/μL but normalized at age 6. His serum IgG levels decreased to <500 mg/dL since the age of 13 years.

Patient 5.2 is a 14-year-old boy with IKAROS HI. He developed SLE at age 3 years. At SLE diagnosis, hypergammaglobulinemia was present (IgG, 2,329 mg/dL; IgA, 154 mg/dL; and IgM, 97 mg/dL) with several positive autoantibodies, including antinuclear, antidouble-stranded DNA, anticardiolipin and antiribonucleoprotein antibodies. Disease activity in this patient was severe, and he required methylprednisolone pulse and immunoadsorption; however, he subsequently received prednisolone for 7 years without showing SLE-related symptoms. Hypogammaglobulinemia was detected at the same time as the SLE symptoms disappeared. He has been in clinical remission for 3 years without SLE treatment.

Patient 11.1 initially presented with enteropathy at the age of 10 years. He had hypogammaglobulinemia and started immunoglobulin replacement therapy when he was 24 years old. He had enteropathy and developed additional complications of type 1 diabetes and CNS involvement in his 30s. Additionally, he developed gastric adenocarcinoma at 34 years, which led to his death from sepsis 2 years after gastrectomy.

Patient 16.2 presented with pneumonia and hypogammaglobulinemia at the age of 18 years, he also had alopecia. He developed GLILD and showed CNS involvement after 2 years. Because those complications were refractory to glucocorticoids, immunosuppressant agents, and abatacept, he received HSCT at 21 years of age. Unfortunately, he died of HSCT-related complications 11 months after HSCT.

Clinical Manifestations and Course

Patient characteristics are presented in **Table 2**. In our cohort, four patients with CTLA4 deficiency underwent HSCT. These patients were evaluated before undergoing HSCT as the procedure could replace the immune system. The median age

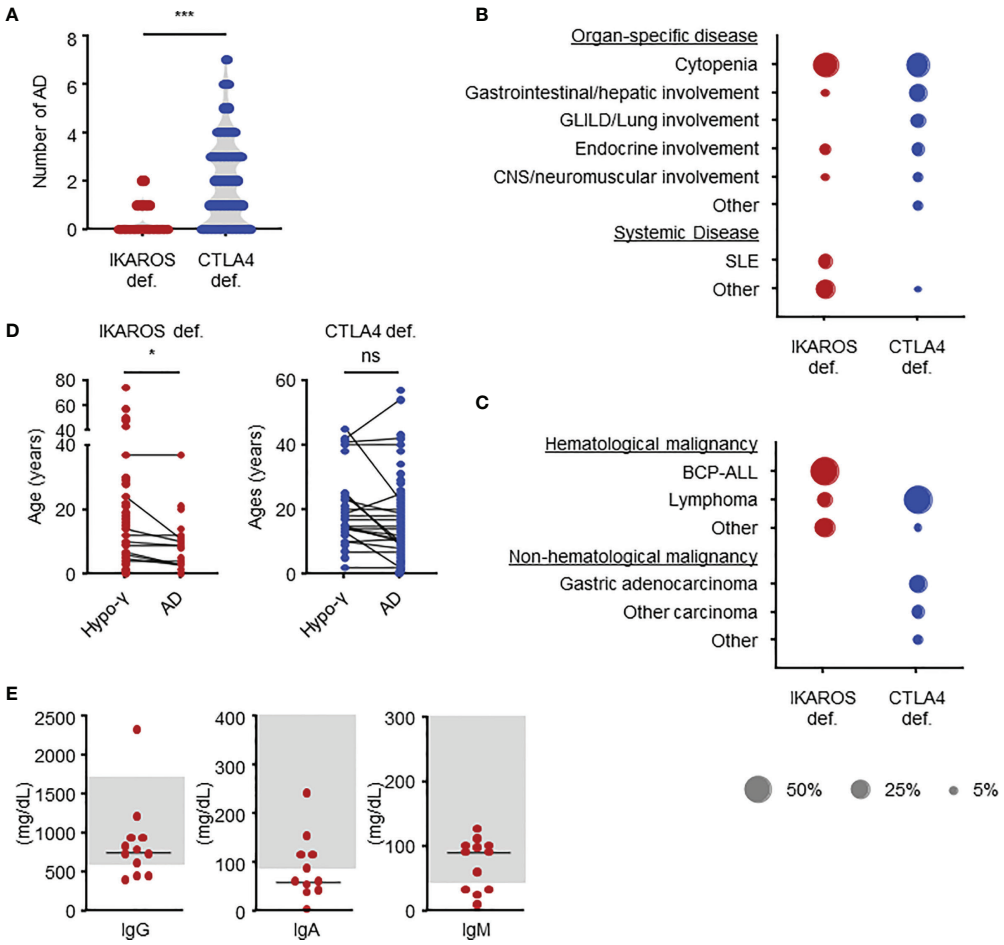

FIGURE 2 | Clinical manifestations in IKAROS and CTLA4 deficiencies as determined by systemic literature reviews. **(A)** Number of autoimmune diseases. **(B)** Types of autoimmune diseases in terms of percentage of total number. **(C)** Types of malignancies. **(D)** Comparison of age at the onset. **(E)** Serum immunoglobulin levels at the onset of autoimmune disease in patients with IKAROS deficiency. The groups were compared using Mann-Whitney *U*-test in **(A)** and Wilcoxon signed-rank test **(D)**. $^{*}P < 0.05$, $^{***}P < 0.001$. AD, autoimmune disease; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; hypo-γ, hypogammaglobulinemia; ns, not significant; SLE, systemic lupus erythematosus.

at last follow-up was 18 (15-41) years for patients with IKAROS deficiency and 29 (20-47) years for those with CTLA4 deficiency. Hypogammaglobulinemia and AD were identified in 9 (60%) and 7 (47%) patients with IKAROS deficiency, and 15 (48%) and 24 (77%) patients with CTLA4 deficiency (**Figure 4B**). More than half of these patients developed both complications. Consistent with the results of the systematic literature reviews, the number of ADs per patients was higher in CTLA4 deficiency (**Figures 4C, D**).

Among the 9 and 15 patients with hypogammaglobulinemia in IKAROS and CTLA4 deficiency conditions, respectively, only one patient with CTLA4 deficiency (patient 15.1) achieved remission (**Figure 4E**). Patient 15.1 had mild hypogammaglobulinemia (IgG, 456 mg/dL; IgA, 95 mg/dL; and IgM, 35 mg/dL) but it improved after starting abatacept treatment. Moreover, 4 (50%) of 8 patients with ADs and 18 (32%) of 57 with ADs achieved remission with IKAROS and CTLA4 deficiencies, respectively (**Figure 4E**).

The number of patients with AD onset and remission was compared between before and after hypogammaglobulinemia onset using person-year method. The onset of AD per 1 person-year, which is the sum of the patient follow-up duration, was highest in CTLA4 deficiency after the onset of hypogammaglobulinemia. However, it was higher before the onset of hypogammaglobulinemia in IKAROS deficiency (**Figure 4F**). Alternatively, the number of remission of AD per 1 person-year, which is the sum of the patient AD duration, was highest in IKAROS deficiency before the onset of hypogammaglobulinemia, whereas remission was achieved in a few patients with CTLA4 deficiency (**Figure 4F**).

DISCUSSION

We performed systematic literature reviews of IKAROS and CTLA4 deficiencies and revealed a possibility that the onset of

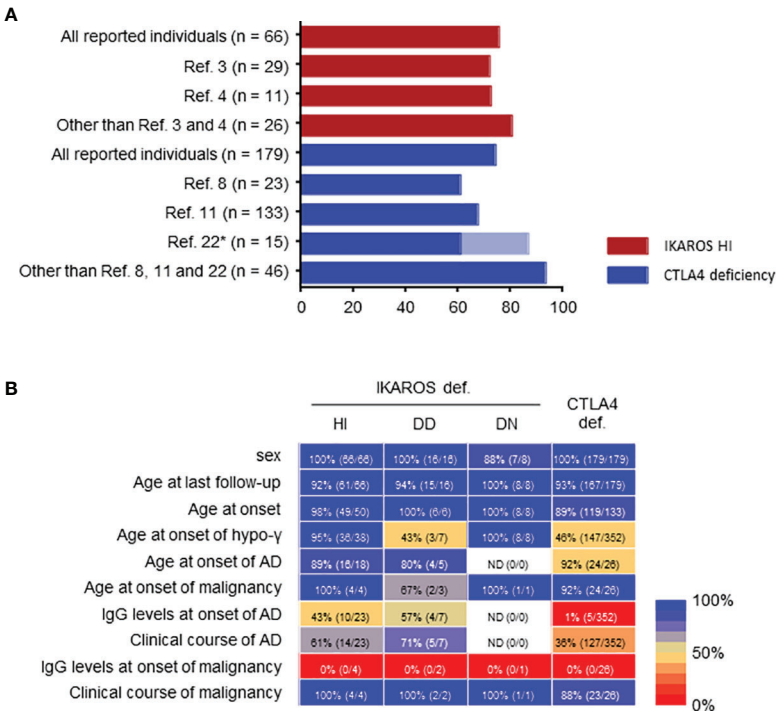

FIGURE 3 | Limitations of systemic literature reviews. **(A)** Penetrance in IKAROS HI and CTLA4 deficiencies. *Penetrance was described as 86.7% (13/15) in ref. 22, but re-evaluated as 60% (9/15) in ref.11 in the same patients. **(B)** Percentage of clinical data available presented as a heatmap: blue, higher percentage available; red, lower percentage available. If even a few data were described, it was considered as available. AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

AD was earlier than that of hypogammaglobulinemia in IKAROS deficiency. Furthermore, we demonstrated the more frequent remission of AD before the onset of hypogammaglobulinemia in IKAROS deficiency in this the retrospective longitudinal study. However, such observations were not found in CTLA4 deficiency (**Figure 5**). Several longitudinal studies of IEI have been described, evaluating clinical presentations, quality of life, and psychological status (24, 25). However, to the best of our knowledge, this is the first longitudinal study to focus on the clinical course of complications in IKAROS and CTLA4 deficiencies over time.

The cumulative incidences of hypogammaglobulinemia and AD are 67%-86% and 3%-33% in IKAROS deficiency and 20%-71% and 61%-86% in CTLA4 deficiency, respectively (3, 4, 7, 8, 23). Most of the previous studies showed a cumulative incidence. However, attention should be paid to the incidence when applying it in clinical settings. It does not reflect disease activity or remission, and patients who remain asymptomatic because of their young age are not considered. Furthermore, that incidence cannot be compared between cohorts of patients of different ages. Previous studies showed various cumulative incidences even in the same disease. However, longitudinal evaluation could provide more useful information in the clinical setting. Our study indicated that the onset of AD was more frequent before that of hypogammaglobulinemia in IKAROS deficiency and after hypogammaglobulinemia onset in CTLA4 deficiency. This observation agrees with the result of the systematic literature review, showing no increase in the cumulative incidences of AD at older ages in IKAROS deficiency. This also agrees with the higher number of AD per patient in CTLA4 deficiency. Furthermore, AD remission was more frequent before the onset of hypogammaglobulinemia in IKAROS deficiency but not in CTLA4 deficiency. These observations support the fact that patients with IKAROS deficiency develop AD and hypogammaglobulinemia in that order and achieve AD remission before hypogammaglobulinemia onset. However, patients with CTLA4 deficiency are more likely to develop AD with increasing age than with developing hypogammaglobulinemia.

Our study clarified the problems of literature reviews and cross-sectional studies, and highlighted that change in clinical manifestations over time had not been noticed until now, despite useful information in clinical settings. Our longitudinal study of the small cohort revealed a new feature that large cross-sectional studies would not reveal. Longitudinal studies are useful in evaluating the clinical picture of diseases with a few patients, such as IEI. Notably, these results do not deny the significance of literature reviews and cross-sectional studies. Each has its strengths and limitations, and a further understanding of the diseases can be obtained by their combination (**Supplemental Table 1**).

Our study also provides an important suggestion for AD pathogenesis. AD pathogenesis in IKAROS deficiency remains unclear. Studies in mice have shown that impaired Ikaros function can cause B-cell hyperactivation, upregulation of inflammatory gene programs in T cells, and impaired Treg

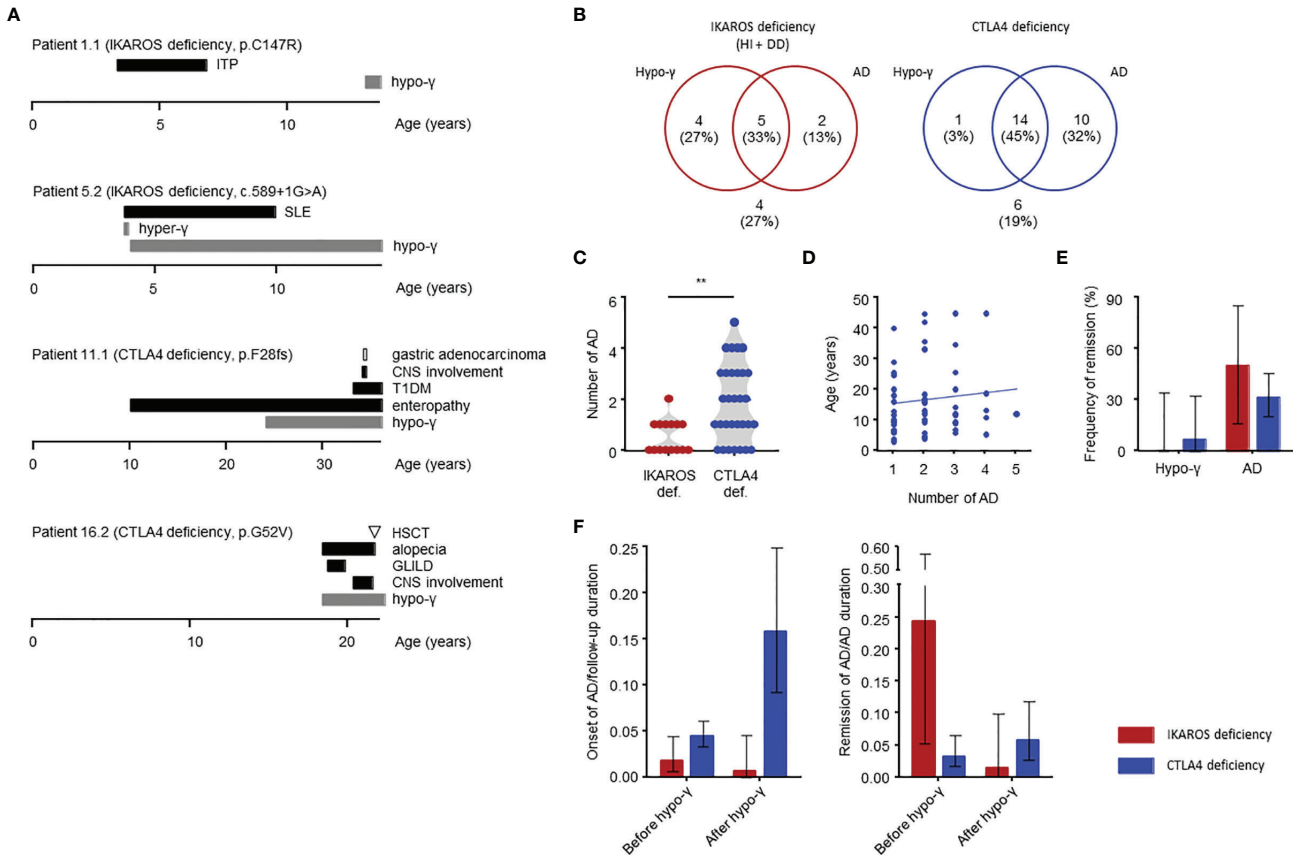

FIGURE 4 | Clinical manifestations in IKAROS and CTLA4 deficiencies determined by longitudinal study. **(A)** Case reports of four patients. The AD duration is defined as from onset to the end of treatment with no symptoms or laboratory abnormalities. CNS, central nervous system; GLILD, granulomatous, and lymphocytic interstitial lung disease; HSCT, hematopoietic stem cell transplantation; hyper-γ, hypergammaglobulinemia; SLE, systemic lupus erythematosus; T1DM, type 1 diabetes mellitus. **(B)** Distribution of complications. **(C)** Number of autoimmune diseases. **(D)** Total number of autoimmune disease and age at onset for CTLA4 deficiency. **(E)** Frequency of remission of hypogammaglobulinemia and autoimmune disease. **(F)** Incidence of autoimmune disease onset or remission before and after onset of hypogammaglobulinemia shown as per 1 person-year. The person-year is the sum of the patient follow-up deration (years) for the onset, and the sum of the patient autoimmune disease duration (years) for the remission. The groups were compared using a Mann-Whitney *U*-test in **(C)**. $^{**}P < 0.01$. Thin bars are presented as 95% CIs in **(E, F)**. AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

function, potentially leading to AD (26–28). The evidence shown in this study suggest a prominent role of B cells in AD associated with IKAROS deficiency. The high incidence of systemic AD and detectable autoantibodies and amelioration of AD after hypogammaglobulinemia onset was shown. This hypothesis is also supported by a case report with IKAROS deficiency-associated ITP and autoimmune hepatitis successfully treated by depleting B cells with rituximab (29). Furthermore, B-cell hyperactivation has been described in humans with IKAROS deficiency and mouse models (30). Mice with specific *Ikzf1*

TABLE 2 | Baseline description of individuals with IKAROS deficiency (HI and DD) and CTLA4 deficiency in the longitudinal study.

	IKAROS HI and DD	CTLA4 def.	*P*-value
Number of patients	15	31	
Sex (M/F)	8/7 (n = 15)	14/17 (n = 31)	0.603
Age at last follow-up	18 [15-41] (n = 15)	29 [20-47] (n = 31)	0.160
Age at onset	10 [4-11] (n = 12)	10 [9-20] (n = 25)	0.060
Age at onset of hypo-γ	10 [9-12] (n = 9)	24 [14-35] (n = 15)	**0.005**
Age at onset of AD	5 [4-18] (n = 7)	10 [9-19] (n = 24)	0.310
Age at onset of malignancy	(n = 0)	31 (n = 2)	

The median ages are shown [with 25th and 75th percentiles] (year).
Bold number indicates the statistically significant correlation.
AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

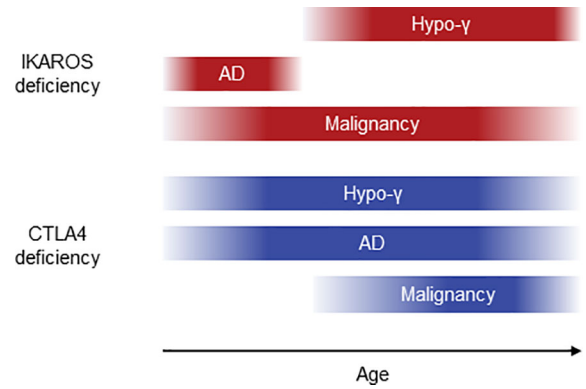

FIGURE 5 | Schematic diagram of clinical courses in IKAROS and CTLA4 deficiencies. AD, autoimmune disease; hypo-γ, hypogammaglobulinemia.

deletion in mature B cells, which develops an autoimmune phenotype, show B-cell hyperactivation due to impaired regulation of B-cell receptor anergy and Toll-like receptor signaling (27). Early mouse studies mainly focused on the role of Ikaros in early B-cell development. In humans with IKAROS deficiency, hypogammaglobulinemia also results from impaired early B-cell development (3, 4). However, IKAROS is also involved in B-cell development and function at various stages, suggesting that dysfunction of mature B cells due to IKAROS deficiency could be involved in AD (31). Given this hypothesis, after the progression of hypogammaglobulinemia, early B-cell development arrest causes loss of autoreactive B cells and normal mature B cells, leading to the amelioration of AD. However, we found one patient (patient 8.1) with IKAROS deficiency in our cohort developed rheumatoid arthritis 33 years after hypogammaglobulinemia onset, suggesting additional mechanisms, such as impaired T-cell function as described in mouse models, in addition to impaired B-cell function (26–28). Regarding CTLA4 deficiency, we found a more organ-specific AD and no association between AD remission and hypogammaglobulinemia onset. These observations support a major role of T cells in AD pathogenesis due to impaired Treg function and hyperactivation of effector T cells. Furthermore, our observation of the amelioration of hypogammaglobulinemia after abatacept treatment in patient 15.1 suggests a direct role of activated T cells in the hypogammaglobulinemia associated with CTLA4 deficiency. Although detailed immunological examination, including bone marrow examination, was not performed in this patient, bone marrow infiltration of T cells, which could lead to hypogammaglobulinemia, has been described in previous studies (7, 9).

Given the observations in this study and previous reports, for AD associated with IKAROS deficiency, even refractory AD such as SLE, there is a possibility that treatment is unnecessary after hypogammaglobulinemia onset. B-cell depletion therapy, such as rituximab, may be a good therapeutic option. As described in SLE, belimumab, daratumumab, or CD19-targeted chimeric antigen receptor T-cell therapy might also be useful (32, 33). For AD associated with CTLA4 deficiency, it is necessary to decide the timing of abatacept or HSCT in considering possible treatment-refractory or potential additional AD, as described in a retrospective world survey (9).

Although the reason is unknown, various clinical manifestations can be observed in patients with IKAROS deficiency, including hypogammaglobulinemia, AD, malignancy, and pancytopenia (3, 4). The genotype-phenotype relationship is not established, except for DN N159S and N159T variants, which cause early-onset combined immunodeficiency (5). AD has not been reported in patients with IKAROS DN, which is likely due to severe B-cell deficiency early after birth. We have previously reported patient 6.1 with the Y210C variant, who had transient pancytopenia early after birth (4). We followed up with this patient up to 6 years of age without additional complications in this study. We also found one patient with the R143W variant (patient 10.1). This variant has been reported in two unrelated families, but its effect is controversial (34, 35). One variant was observed in four patients who presented with hypogammaglobulinemia, AD, or both, described as HI (34). Another was in a patient with hypogammaglobulinemia, autoimmune hemolytic anemia, and pancytopenia, described as partial DN (35). However, our patient presented with SLE and APS, typical manifestations as HI. We also found one patient with the R143W variant who presented with hypogammaglobulinemia in our other French cohort. Therefore, from our clinical observations, the R143W variant seems to behave as an HI variant rather than partial DN. The future challenge for IKAROS deficiency is to uncover the clinical course over time and its mechanisms in each variant.

Conclusively, this study provided more realistic manifestations of IKAROS and CTLA4 deficiencies that suggest their pathogenesis. The establishment and use of disease registries and databases will lead to the higher quality of clinical studies and further understanding of human diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Tokyo Medical and Dental University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AH did conception and design. ET, KO, MiY, KK, GY, DK, YY-S, JK, YO, TaI, MN, MaY, KT, ToI, KM-S, YS, TD, TY, YN provided clinical information. AH performed experiment. AH analyzed data and wrote the manuscript. NM, MoY, MT, KI, SN, TM provided critical discussion. SL and HK supervised the study and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.784901/full#supplementary-material

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Primary Immunodeficiencies Associated With Early-Onset Inflammatory Bowel Disease in Southeast and East Asia

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Background: Causes of early-onset inflammatory bowel disease (IBD) vary, and primary immunodeficiency diseases (PIDs) are associated with early-onset IBD as monogenic disorders.

Aim: This review investigates the prevalence, clinical manifestation, genetic profile, and treatment of patients with early-onset IBD in Southeast and East Asia.

Methods: A systemic review of articles reporting PID patients associated with early-onset IBD in Southeast and East Asia was conducted.

Results: The prevalence of PID associated with IBD was higher than that reported in western nations, and the frequency of patients with bloody stools as an early symptom was relatively higher in monogenic diseases. A total 13 (12.0%) of 108 patients with early-onset IBD were diagnosed as PID by exome sequencing and targeted gene panel analysis in Japan, including four patients with *XIAP*, three with *IL10RA*, and two or one patient with other gene mutations. In addition, ten patients were reported as having IL-10 receptor alpha (IL-10RA) deficiency in China and Hong Kong. Allogeneic hematopoietic stem cell transplantation was performed in patients with X-linked inhibitor of apoptosis deficiency, IL-10RA deficiency, or other PID as a curative treatment, and the preferable outcome of reduced-intensity conditioning and complete resolution of IBD symptoms and dysbiosis were achieved.

Conclusion: Comprehensive molecular diagnosis has been widely applied to screen for patients with PID-associated IBD in Southeast and East Asia. These results contributed to the awareness of monogenic PID in early-onset IBD patients and their differences in clinical manifestations and genetic profiles compared to the patients in western counties.

Keywords: early-onset inflammatory bowel diseases, primary immunodeficiency diseases, exome sequencing, allogeneic hematopoietic stem cell transplantation, IL-10RA deficiency, XIAP deficiency

INTRODUCTION

Inflammatory bowel disease (IBD) is caused by various factors, including genetic background, host-microbe interactions, dysbiosis, and environmental factors (1, 2). Recent comprehensive genome-wide studies revealed that some patients with IBD have disease-causing mutations or single-nucleotide polymorphisms that increase the risk of IBD in adults (3). Pediatric patients with early-onset IBD (EO-IBD) currently defined as clinical manifestations and/or being diagnosed under the age of 10 years old, including with very early-onset IBD (VEO-IBD) under the age of 6 years old, have distinct clinical features from those of adult patients with IBD, and some of the patients show an unclassified histology distinct from that of classical ulcerative colitis (UC) or Crohn's disease (CD).

The worldwide VEO-IBD consortium, created in 2014, has contributed to understanding the molecular basis of VEO-IBD and to the development of personalized treatments for patients with these rare diseases (4). It has been reported that genes responsible for primary immunodeficiency diseases (PID), including interleukin-10 (IL-10) or IL-10 receptor (IL-10R) deficiency, X-linked inhibitor of apoptosis (XIAP) deficiency, immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, Wiskott–Aldrich syndrome (WAS), chronic granulomatous disease (CGD), and common variable immunodeficiency, are involved in the molecular pathogenesis of pediatric IBD (5–7). Diagnostic approaches using exome sequencing and targeted gene panel analysis have contributed to the definite molecular diagnosis of early-onset IBD (8–12). Crowley et al. (2020) reported the prevalence and clinical symptoms of early-onset IBD patients enrolled from worldwide countries. They identified 40 rare variants associated with 21 disease-causing genes in 31 (3.1%) of 1,005 patients with IBD. These variants occurred in 7.8% of IBD patients younger than 6 years old and in 2.3% of children aged 6–18 years old. Of the 17 patients with monogenic CD, 35% experienced abdominal pain, 24% had non-bloody loose stool, 18% had vomiting, 18% had weight loss, and 5% had intermittent bloody loose stool. Of the 14 patients with monogenic UC or unclassified histology, their most predominant feature was bloody loose stool (78%). Twenty-two patients (2.2%) had variants in genes responsible for PID, including five variants in *XIAP;* three in *DOCK8;* two each in *FOXP3*, *LRBA* and *ARPC1B;* and one each in *IL10RB*, *CYBB*, and other genes. Only 1% of the patients with variants were considered potential candidates for correction of variants by allogeneic hematopoietic stem cell transplantation (HSCT) (13).

In accordance with the worldwide recognition, multicenter studies for pediatric patients with IBD have been reported in Japan, China, Hong Kong, and Malaysia in recent years. This review of relevant published literatures in the Southeast and East Asia summarizes the prevalence, clinical manifestations, results of genetic analysis by exome sequencing and targeted gene panel analysis, and treatment options of allogeneic HSCT as a curative therapy focusing on IL-10RA deficiency, XIAP deficiency and other PID.

METHODS

A comprehensive search of articles reporting PID patients associated with early-onset IBD in Southeast and East Asia was performed using PubMed (http://pubmed.ncbi.nlm.gov). The following search terms or abbreviations were used: primary immunodeficiency, PID, inflammatory bowel disease, IBD, early-onset IBD, VEO-IBD, Asia.

The present study was approved by the Ethics Committee of the Tohoku University Graduate School of Medicine.

PREVALENCE AND CLINICAL MANIFESTATIONS

Lee et al. (2016) described the prevalence and clinical features of VEO-IBD in University Malaya Medical Center, Malaysia. Six patients (13%, CD = 3, UC = 2, IBD-unclassified = 1) out of 48 pediatric patients (CD = 25, UC = 23) of IBD were infantile-onset IBD before 12 months of age. Compared with later-onset IBD patients, infantile-onset IBD patients were more likely to present with bloody diarrhea; however, no mutation in IL-10 or IL-10R was identified in enrolled patients (14). Ishige et al. (2010) analyzed a national IBD registry database of Japanese patients treated between 2003 and 2006, and reported that 10.6% of CD patients and 5.9% of UC patients were under the age of 16 years old. They showed that pediatric patients with IBD had clinical features that are distinct from those in adult patients with IBD. In comparison with adults, pediatric patients more commonly had a positive family history of CD and UC, tended to have more severe disease, and more often had extensive colitis in UC (15). Maisawa et al. (16) retrospectively investigated the clinical features of children who were diagnosed with IBD between 1998 and 2008 in Japan, especially those whose onset were younger than 8 years of age. Totally, 24 patients with CD and 47 patients with UC were analyzed on the basis of the final diagnosis. Among patients with CD, the age at onset was less than 1 year in 62.5% of patients; 87.5% of CD patients involved the colon; and 63.8% of UC cases were pancolitis. Growth failure was more severe at diagnosis in CD patients than in UC patients. Familial occurrence within first-degree relatives was observed in eight families among 45 patients with UC, compared with none among CD patients. They indicated that the prevalence and clinical manifestations of IBD in infants and children in Japan differed from that in western countries in terms of earlier age at disease onset of CD and higher incidence of familial occurrence of UC (16). Suzuki et al. (17) analyzed 35 Japanese patients under 16 years of age who were suffering from severe and refractory IBD and were enrolled in this multicenter study, including 27 patients with VEO-IBD under 6 years of age. In total, 5 of 35 patients (14.3%), including 4 patients with VEO-IBD, were diagnosed with monogenic disorders (17). Subsequently, Uchida et al. (18), in the same institute analyzed 108 enrolled patients under 17 years of age who were suffering from early-onset diarrhea and were refractory to conventional therapies in Japan. They reported that a family history of Bechet's disease was predominantly related to monogenic

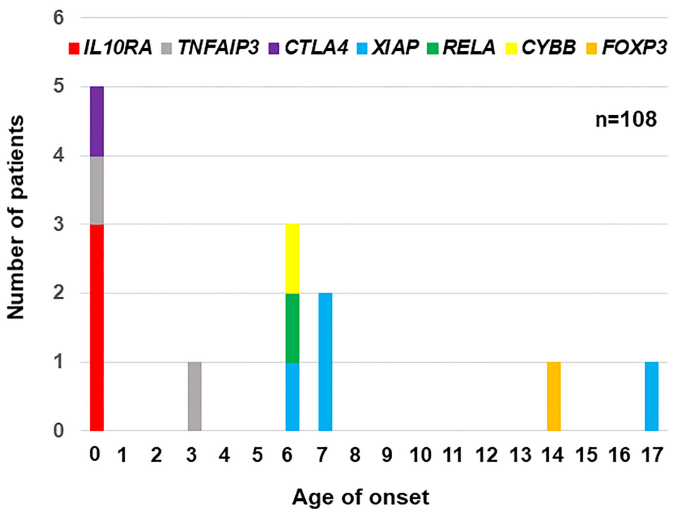
disease and that the frequency of patients with bloody stools as an early symptom was relatively higher in monogenic disease than in non-monogenic cases (18).

GENETIC PROFILES

We established exome sequencing and targeted gene panels covering all responsible genes for PID and early-onset diarrhea. The data revealed that a total of 15 (13.9%) out of 108 patients enrolled in the study were monogenic. A total of 13 (12.0%) patients were diagnosed as monogenic PID in Japan (17, 18), and the frequency of monogenic PID among early-onset IBD patients was relatively higher than that of 2.2% among 1,008 early-onset IBD patients reported in western counties (13). The different incidence of monogenic IBD between western countries and Japan may be caused by the criteria for enrolled patients, genetic background and other environmental factors. The age distribution of 13 monogenic PID patients in Japan is shown in **(Figure 1A)** and their actual disease-causing mutations in responsible genes for PID are shown in **(Figure 1B)**. The number of patients with gene mutations were four patients with *XIAP*, three with *IL10RA*, two with *TNFAIP3*, and one each with *RELA*, *CTLA4*, *FOXP3*, and *CYBB* gene mutations. All three patients with IL-10RA deficiency were under 1 year of age; all four patients with XIAP deficiency were over 6 years of age at onset. The patient with refractory diarrhea caused by heterozygous truncated RelA protein expression was the first identified case worldwide, and functional analysis revealed that the mutation affected nuclear factor-kappa b (NFκB) signaling. Genotypes were significantly associated with clinical and pathological findings in each patient. Yanagi et al. (21) and Ishige et al. (22) reported another patient with IL-10RA deficiency. Ishihara et al. (23) reported a rare patient with Hermansky–Pudlak syndrome in Japan (23).

Ten patients with IL-10RA deficiency were reported in Hong Kong and China as listed in **Figure 1C**. Mao et al. (2012) in Hong Kong identified a patient with compound heterozygous mutations in the *IL10RA* gene in 2012 (24). Peng et al. (2018) in China diagnosed up to nine patients with IL-10RA deficiency by exome sequencing and treated them using reduced-intensity conditioning (RIC) followed by umbilical cord blood transplantation (CBT) (19). All ten patients showed initial manifestations within one month after birth and had a common mutation of c.C301T (p.R101W) in *IL10RA* gene. These findings indicate that the frequency of IL-10RA deficiency in China is higher than in western countries. Moreover, predictive prenatal diagnosis for IL-10RA deficiency in eight families was performed in China, although the legal and ethical considerations are still controversial for prenatal diagnoses of diseases for which curative treatments are available after birth (24).

A



B

Age of onset	Sex	Gene mutation	Inheritance
0m	F	IL10RA:exon3:c.G350A:p.R117H IL10RA:exon5:c.C634T:p.R212X	Autosomal recessive
0m	M	IL10RA:exon4:c.G537A:p.T179T	Autosomal recessive
3m	M	IL10RA:exon4:c.G537A:p.T179T	Autosomal recessive
3m	F	TNFAIP3:exon4:c.487-1G>A	Autosomal dominant
3y	M	TNFAIP3:exon4:c.610delA:p.R204fs	Autosomal dominant
0m	M	CTLA4:exon2:c.137dupT:p.V46fs*14	Autosomal dominant
6y	M	XIAP:exon6:c.T1141C:p.R381X	X-linked
7y	M	XIAP:exon2:c.387_390del:p.L129fs	X-linked
7y	M	XIAP:exon2:c.650delG:p.W217fs	X-linked
17y	M	XIAP:exon2:c.C847T:p.G283X	X-linked
6y	M	RELA:exon11:c.C1165T:p.Q389X	Autosomal dominant
6y	M	CYBB:exon6:c.T615A:p.F205L	X-linked
14y	M	FOXP3:exon11:c.T1099C:p.F367L	X-linked

C

Age of onset	Sex	Gene mutation Common mutation in all patients: IL10RA:exon3:c.C301T:p.R101W	Number of patients
0m	M	IL10RA:exon3:c.C251T:p.T84I	1
0m	F	IL10RA:exon3:c.T299G:p.V100G	1
0m	F	IL10RA:exon3:c.C301T:p.R101W	1
0m	M	IL10RA:exon3:c.C301T:p.R101W	1
0m	F	IL10RA:exon4:c.G537A:p.T179T	4
0m	M	IL10RA:exon4:c.G537A:p.T179T	2

FIGURE 1 | A summary of 23 patients with PID-associated early-onset IBD in East Asia. **(A)** Age distribution of disease onset according to the responsible genes in 13 Japanese patients. **(B)** A list of disease-causing mutations in responsible genes for PID in 13 Japanese patients (17, 18). **(C)** A list of disease-causing mutations in 10 patients with IL-10RA deficiency reported from Hong Kong (19) and China (20). A synonymous p.T179T variant caused a splicing error in *IL10RA* gene. IL10RA, interleukin-10 receptor subunit alpha; TNFAIP3, tumor necrosis factor alpha-induced protein 3; CTLA4, cytotoxic T-lymphocyte-associated protein 4; XIAP, X-linked inhibitor of apoptosis protein; RELA, v-rel avian reticuloendotheliosis viral oncogene homolog A; CYBB, cytochrome b beta; FOXP3, forkhead boxprotein P3; M, male; F, female.

Kammermeier et al. (2014) and Uhlig et al. (2021) proposed integrated diagnostic methods and clinical genomics for IBD-related PID patients based on their different implications in systemic immunity (11, 12). Genetic profiles in most of IBD-related PID patients in East Asia can be sorted into the algorithms, although molecular pathogenesis of a patient with a heterogenous *RELA* mutation remained to be clarified.

TREATMENTS

Genetic investigations provided us a new approach for better treatments other than immunosuppressive agents, nutritional supplement and surgical intervention. One of the curative treatments for PID with early-onset IBD is allogeneic HSCT, and RIC is a suitable treatment option for these nonmalignant diseases in terms of reduced long-term sequelae if engraftment and complete chimera are achieved after HSCT. Allogeneic HSCT can induce remission in patients with IL-10 and/or IL-10R deficiency (25, 26). In Japan, only two patients with IL-10RA deficiency were reportedly treated successfully with RIC followed by allogeneic HSCT and achieved disease remission after engraftment (21, 27). In China, nine patients with severe clinical conditions with IL-10RA deficiency received RIC followed by allogeneic CBT, and six patients achieved complete remission without evidence of graft-vs.-host disease (GVHD) or infections. However, one patient died of chronic lung GVHD at 6 months post-transplantation, and the other two patients died of sepsis due to unsuccessful engraftments. Severe malnutrition and growth retardation associated with the disease were significantly improved in engrafted patients (19). In addition, Neven et al. (2013) reported a Mendelian predisposition to B-cell lymphoma caused by IL-10R deficiency, and allogeneic HSCT was a curative treatment for the life-threatening complication (28). Therefore, optimal RIC regimens for successful engraftment should be discussed in IL-10RA deficiency.

Allogeneic HSCT has also been applied for IBD in XIAP deficiency as a curative treatment option (29–31). Yang et al. (2012) reported clinical and genetic characteristics of XIAP deficiency in Japan, and established flowcytometric analysis of XIAP protein expression in lymphocytes, which was useful for determining engraftment and chimeric status in each hematopoietic cell lineage (32). Ono et al. (2017) reported a nationwide survey of details of allogeneic HSCT for XIAP deficiency in Japan. They showed that the optimal RIC regimen containing hemophagocytic lymphohistiocytosis control by etoposide and dexamethasone palmitate might be important factors for successful outcomes and improved IBD symptoms in patients with XIAP deficiency. Nine of the 10 patients were alive and well at a median of 21.2 months after HSCT (33). They recently reported that IBD associated with XIAP deficiency is caused by dysbiosis of the gut microbiota, and allogeneic HSCT ameliorated gut inflammation and dysbiosis in patients with XIAP deficiency (34). Amelioration of IBD symptoms is expected to improve the quality of life of patients treated with HSCT. Our endoscopic and histological findings revealed that colon symptoms significantly ameliorated after HSCT in Japanese patients with XIAP deficiency (**Figure 2**).

Other PID-associated IBDs included IPEX syndrome caused by *FOXP3* gene mutation and the absence of regulatory T cells (Tregs). Horino et al. (2014) reported a selective expansion of donor-derived Tregs after allogeneic bone marrow transplantation in a mixed chimeric state in a patient with IPEX syndrome, thus suggesting a growth advantage of normal Tregs in the IPEX patient. He suffered from severe diarrhea and required parenteral nutrition and red blood cell transfusion because of severe bloody diarrhea. The gastrointestinal symptoms were ameliorated after engraftment and expansion of CD4+CD25+Foxp3+ Tregs. However, optimal conditioning regimens should be further discussed to achieve a complete chimeric state in the rare patients with IPEX syndrome (35).

If the patients had no human leukocyte antigen (HLA)-identical donors or suitable cord blood for transplantation, an HLA haplo-identical related donor was another candidate for HSC sources. Osumi, et al. (2020) reported a retrospective study of allogeneic HSCT with post-transplantation cyclophosphamide and antithymocyte globulin from HLA-mismatched related donors for nonmalignant diseases, and XIAP deficiency, IL-10RA deficiency, WAS and CGD patients were included in the case series (27).

In terms of treatments other than HSCT, the frequency of patients treated with gastrointestinal surgery was relatively

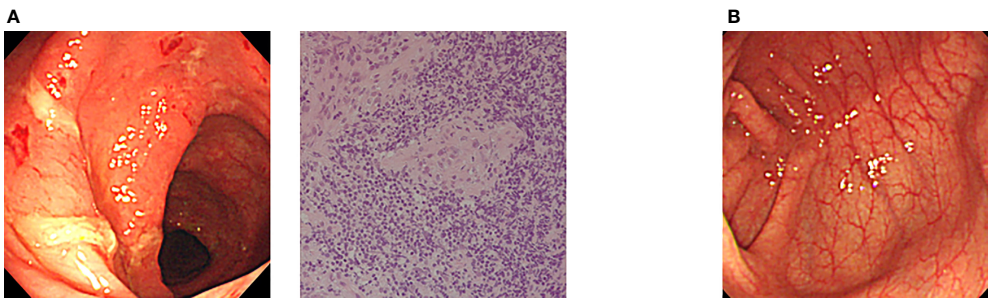


FIGURE 2 | Amelioration of endoscopic findings after allogeneic CBT in a Japanese patient with XIAP deficiency. **(A)** Endoscopic evaluation at onset showed edema, hemorrhage and ulcer formation. Pathological evaluation of biopsy specimen at onset showed nonspecific active inflammation. **(B)** Completely ameliorated endoscopic finding after allogeneic CBT in the same patient (17, 33).

higher in the monogenic patients than in non-monogenic IBD patients (15–18). The relationship between the usage rate of prednisolone (PSL) or anti-TNF-α antibody and the monogenic disease was not clear in Japan. PSL was effective for 55.1% of non-monogenic and 40.0% of monogenic IBD patients. The anti-TNF-α antibody was effective for 69.4% of non-monogenic and 37.5% of monogenic IBD patients, suggesting that the refractory properties of the anti-TNF-α antibody were conspicuous in monogenic patients. As one of other therapeutic options, the effective treatments with an abatacept, a fusion protein of the extracellular domain of the human cytotoxic T-lymphocyte-associated protein 4 (CTLA4) linked to a modified Fc of human IgG1, were reported in patients with monogenic IBD caused by lipopolysaccharide (LPS)-responsive and beige-like anchor (LRBA) deficiency and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) deficiency in China (36) and worldwide (37).

IMPLICATION FOR IMMUNITY IN GASTROINTESTINAL TRACT

The genes responsible for PID play critical roles in normal immunity in the gastrointestinal tract. Using whole-exome sequencing and targeted gene panel analysis, they identified underlying gene mutations responsible for PID in pediatric patients with early-onset IBD in an East Asian population. We are aware of the evidence that normal IL-10 signaling, nucleotide-binding oligomerization domain-containing protein 2 (NOD2)-mediated signaling and Tregs play indispensable roles in keeping normal immune homeostasis in the gastrointestinal tract by determining immunological defects in patients with IL-10 signaling deficiency, XIAP deficiency and IPEX syndrome, respectively.

Classical WAS patients also suffer from VEO-IBD (38, 39). A complex of Wiskott-Aldrich syndrome protein (WASP) and WASP-interacting protein (WIP) is recruited to the immunological synapse between antigen presenting cells and T cells, regulating T cell receptor-driven IL-2 production and actin polymerization in T cells (40–42). A fraction of WASP is localized in the nucleus and loss of WASP induces impaired T helper 1 (Th1) cell functions and Th2-dominant immunity (43). Nguyen et al. (2007) reported that a relative Th2 cytokine predominance is critical for the colitis in WASP-deficient mice (44). They also reported that defective interactions between WASP-deficient innate immune cells and T cells induced the dysfunctions of tolerogenic dendritic cells, impaired IL-10 signaling and homeostasis of Tregs in mouse model (45). Therefore, Th2-colitis, defective IL-10 signaling and Tregs are involved in the pathogenesis of colitis in WAS patients.

We recently reported that a pig model of X-lined severe combined immunodeficiency (X-SCID) completely lacked Peyer's patches and IgA production in the small intestine. Allogeneic HSCT to X-SCID pigs did not facilitate the lymphoid organogenesis completely and created atypical intestinal immune and microbial environments in the animal model of X-SCID. We also showed that our patients with X-SCID in mixed chimera after current allogeneic HSCT showed lower IgA levels and dysbiosis in their stool samples compared to the patients in complete chimera or normal individuals, indicating that common γ chain had significant roles in intestinal lymphoid organogenesis (46).

CONCLUSION

Comprehensive molecular diagnosis has been widely applied to screen monogenic PID with early-onset IBD patients in Southeast and East Asia. The findings of different studies and contrasting issues compared to western counties are relatively higher frequency of IBD patients associated with various PID in Japan and higher frequency of IL-10RA deficiency in China and Hong Kong. Identifying links between genetic mutations and clinicopathological and immunological parameters helped us understand the pathogenesis and select appropriate therapies, such as infliximab, immunosuppressive therapy, or allogeneic HSCT as a curative treatment for patients with IBD. Determining whether unidentified genes, a dysregulated immune response to intestinal microbiota, or dysbiosis are involved in the pathogenesis of these diseases may expand our understanding of normal immunity and host-microbe interactions in the gastrointestinal tract.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to this work and approved it for publication.

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WASp Deficiency Selectively Affects the TCR Diversity of Different Memory T Cell Subsets in WAS Chimeric Mice

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Background: The T cell receptor (TCR) diversity is essential for effective T cell immunity. Previous studies showed that TCR diversity in Wiskott–Aldrich Syndrome (WAS) patients was severely impaired, especially in the memory T cell populations. Whether this defect was caused by intrinsic WASp deficiency or extrinsic reasons is still unclear.

Methods: We sorted different T cell subsets from the bone marrow chimeric mice model using both magnetic beads and flow cytometry. TCR repertoires of memory T cells, especially $CD4^+$ effector memory T (TEM) cells and $CD8^+$ central memory T (TCM) cells, were analyzed using the UMI quantitative high-throughput sequencing (HTS).

Results: An average of 5.51 million sequencing reads of 32 samples was obtained from the Illumina sequencing platform. Bioinformatic analyses showed that compared with wild type (WT), WAS knock out (KO)-$CD4^+$ TEM cells exhibited increased Simpson index and decreased D50 index ($P < 0.05$); The rank abundance curve of KO-$CD4^+$ TEM cells was shorter and steeper than that of WT, and the angle of ^qD and q in KO-$CD4^+$ TEM cells was lower than that of WT, while these indexes showed few changes between WT and KO chimeric mice in the $CD8^+$TCM population. Therefore, it indicated that the restriction on the TCRVβ repertoires is majorly in KO-$CD4^+$ TEM cells but not KO- $CD8^+$ TCM cells. Principal Component Analysis (PCA), a comprehensive parameter for TCRVβ diversity, successfully segregated $CD4^+$ TEM cells from WT and KO, but failed in $CD8^+$ TCM cells. Among the total sequences of *TRB*, the usage of TRBV12.2, TRBV30, TRBV31, TRBV4, TRBD1, TRBD2, TRBJ1.1, and TRBJ1.4 showed a significant difference between WT-$CD4^+$ TEM cells and KO-$CD4^+$ TEM cells ($P < 0.05$), while in $CD8^+$ TCM cells, only the usage of TRBV12.2 and TRBV20 showed a substantial difference between WT and KO ($P < 0.05$). No significant differences in the hydrophobicity and sequence length of TCRVβ were found between the WT and KO groups.

Conclusion: WASp deficiency selectively affected the TCR diversity of different memory T cell subsets, and it had more impact on the TCRVβ diversity of $CD4^+$ TEM cells than $CD8^+$

TCM cells. Moreover, the limitation of TCRVβ diversity of $CD4^+$ TEM cells and $CD8^+$ TCM cells in WAS was not severe but intrinsic.

Keywords: Wiskott–Aldrich Syndrome, memory T cell, T cell receptor repertoire, high-throughput sequencing, chimeric mouse model

INTRODUCTION

Wiskott–Aldrich syndrome protein (WASp) is expressed exclusively in the hematopoietic cells, consisting of five main functional domains, a WASp-homology 1/pleckstrin homology (WH1/PH) domain, a basic domain, a GTPase binding domain (GBD), a proline-rich region, and a C-terminal VCA region (a verproline (V) homology domain, a cofilin (C) homology domain; and a central acidic (A) region) which binds the Arp2/3 complex enhancing actin nucleation and rapid formation of new actin filaments (1, 2). As an actin nucleation promoting factor, WASp regulates the structure and dynamics of actin filament networks of the cells (3). The absence or altered structures of WASp result in the Wiskott–Aldrich syndrome (WAS), a rare primary immunodeficiency disease, which is clinically characterized by thrombocytopenia, eczema, immunodeficiency, and increased risk of autoimmune diseases and lymphoid malignancies (4). Indeed, numerous cellular activities of the immune system have been described to be affected in WAS patients, such as reduced chemotactic responses and phagocytic abilities of monocytes and macrophages, impaired activation, differentiation, and proliferation of multiple T and B lymphocyte subsets (4–7). Abnormal T cell functions caused by WASp-deficiency mainly lead to immune deficiency in patients with WAS. The abnormal T cell functions in WAS patients include T lymphopenia, which gradually aggravated with age, decreased immune synapse formation, reduced synthesis, secretion of T cell cytokines (such as IL-2, IFN-γ, and TNF-α), impaired function of cytotoxic T cells, abnormal chemotaxis of T cells *in vitro*, and dysfunction of Treg and regulatory helper T cells (5, 8–11).

T cell receptor (TCR) diversity is an essential guarantee for effective T cell immunity. The TCR repertoire is composed of all TCR clones, in which each TCR clone specifically recognizes the corresponding antigen. The abundance of TCR diversity determines the potential of T cell response to various antigens in the changeable environment. Recombination of Variable (V), Diversity (D), and Joining (J) gene elements allow the establishment of TCR repertoire (12, 13). With the fast-developing next-generation sequencing technology, several studies have explored the role of WASp in the TCR recombination process. In 2005, Wada et al. firstly studied the diversity of TCR in WAS patients and found that TCRVβ repertoire was specifically skewed in WAS patients older than 15 years old (14). Then, Braun et al. and our team confirmed that young WAS patients also had a TCRVβ repertoire defect (15, 16). We further found that the TCR diversity of WAS patients was severely limited in memory/effector $CD4^+$ T cells and terminal effector $CD8^+$ T cells. In contrast, naïve $CD4^+$ T cells and naïve $CD8^+$ T cells showed no limitation on TCR diversity. O'Connell et al. also showed WAS patients had TCR clonal expansion in memory $CD4^+$ T cells, naïve and memory $CD8^+$ T cells (17).

Previous studies have confirmed that the number of TCR clones is affected by many factors, such as age, pathogen infection, tumor, autoimmune diseases, immunization, and immunosuppression (18, 19). Petersen et al. showed that TCR diversity was limited in old $WASp^{-/-}$ mice, but not in young $WASp^{-/-}$ mice. They suggested that autoantigens are likely the cause of reduced TCR diversity in WAS in the absence of infections (20). However, whether the TCR diversity limitation in WAS was caused by intrinsic WASp deficiency is still unclear. Here, we further explored the impact of WASp on TCR diversity of different memory T cell subsets in WAS chimeric mice by unique molecular identifiers (UMI) quantitative high-throughput sequencing (HTS) technology. Our data indicated that WASp deficiency had more impact on the TCRVβ variety of $CD4^+$ TEM cells than that of $CD8^+$ TCM cells. Moreover, the limitation on the TCRVβ diversity of $CD4^+$ TEM cells and $CD8^+$ TCM cells in WAS is not severe but intrinsic. It provides valuable information for unraveling the role of WASp in the TCR recombination process.

MATERIALS AND METHODS

Mouse strains and Chimeric Mice by Bone Marrow Transplantation

Wenxia Song from the University of Maryland kindly provided WASp-KO mice expressing CD45.2 on the C57BL/6 background. WT C57BL/6 mice expressing CD45.1 were purchased from Shanghai Model Organisms. For a generation of bone marrow (BM) chimeras, a total of 5×10^6 BM cells containing WT CD45.1 and WT or $WASp^{-/-}$ CD45.2 at a 1:3 ratio were injected into lethally irradiated (6 Gy) WT CD45.1 recipient animals *via* tail vein. All donor mice were 6–8 weeks old. Chimeric mice were analyzed 10 weeks after transplantation (21). All animal work was reviewed and proved by the Institutional Animal Care and Usage Committee of Children's Hospital of Chongqing Medical University.

Cell Sorting

Purified T cells from chimeric mice were isolated by immunomagnetic negative selection (Stem Cell Technologies, Canada, Cat. 19751). Then, they were stained with the following antibodies: anti-CD3-FITC, anti-CD4-PE/CY7, anti-CD8-APC, anti-CD44-perCP/CY5.5, anti-CD62L-BV421, anti-CD45.1-BV510, and anti-CD45.2-APC-CY7. $CD4^+$ effector memory T cells ($CD45.2^+CD4^+CD44^{hi}CD62L^{low}$) and $CD8^+$ central memory T cells ($CD45.2^+CD8^+$ $CD44^{hi}CD62L^{hi}$) were then sorted using a FACSAria II (BD Biosciences) (**Figure S1**). All antibodies were purchased from BioLegend, USA. The purity of sorted cell subtypes exceeded 95%, as assessed by flow cytometry analysis. **Table S1** lists

the proportions and actual collected cell numbers of the two sorted subsets in each sample.

High-Throughput TCR Repertoire Sequencing and Bioinformatic Analyses

RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturers' instructions and was sent to Huayin Health Technology Co., Ltd. (Guangzhou, China) for HTS analysis employing unique molecular identifiers (UMI) (Publication Patent Number: CN108893464A). RNA concentrations were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA libraries contained UMI for HTS were prepared by 5' rapid amplification of cDNA ends (RACE) using the single primer designed according to the constant region. Then, two rounds of nested PCR were performed for TCRVβ library preparation and the products were purified using QIAquick PCR Purification Kit (Qiagen, Germany). According to the manufacturer's protocol, Illumina adaptors were ligated using the NEBnext Ultra DNA Library Prep kit (New England BioLabs, USA). Then products were identified on 2% agarose gels, and bands centered at 600–800 bp were excised and purified using a QIAquick Gel Extraction kit (Qiagen, Germany). The purified PCR product was subjected to HTS using the Illumina HiSeqX Ten (PE150) and HiSeqX Ten Reagent kit v2.5 (FC-501-2501). Low-quality sequences were discarded. TCRβ V, D, and J gene identification, CDR3 sequence extraction and error corrections in clean reads were performed using miTCR.

Considering the influence of differences in sample size on diversity indices, we randomly sampled 4,000, 6,000, 8,000, 10,000, and 12,000 UMI from each sample for this analysis. Shannon, Simpson (1-D), D50, Chao 1, TOP100, and ^{q}D were assessed based on previously published work (22, 23). Overlap indices were calculated by the overlap coefficient (overlap (X, Y) = |X and Y|/min (|X|, |Y|) for nucleotide sequences (species = nucleotide sequence) (24). TCR CDR3 overlap was assessed by 'F2', 'R' and 'D' metrics in VDJTOOLS software (25). The similarity of CDR3 amino acid was assessed by Bhattacharyya distance as previously described (26). The CDR3 nucleotide length was assessed by Complexity score and Skewness (22). The hydrophobic index was calculated by the frequency of hydrophobic amino acid doublets at positions 6 and 7 of the CDR3β (22, 27). Cysteine index was calculated by the frequency of TCRVβ sequences with cysteine within 2 positions of the CDR3 (27).

Statistical Analysis

The Student's t-test was used to compare diversity parameters in different groups. The Chi-squared test was used to compare groups in analysis involving qualitative variables. The Wilcoxon rank-sum test was used to compare independent samples. Dunnett's multiple comparisons were used for multiple t-tests. Data analysis was performed by GraphPad Prism 7.0 (GraphPad Software, San Diego, CA); p-value <0.05 was considered statistically significant.

RESULTS

To investigate whether WASp creates diverse TCR repertoires in memory T cells independent of the influence from infection and homeostasis, we established BM chimeras of WT (CD45.1) and KO ($WASp^{-/-}$ CD45.2). For HTS, WT or KO $CD4^+$ effector memory T ($CD4^+$ TEM) cells and $CD8^+$ central memory T ($CD8^+$ TCM) cells were sorted 10 weeks after transplantation (21). The proportion and the actual number of collected $CD4^+$ TEM and $CD8^+$ TCM are shown in **Table S1**. Due to a limited cell number, $CD4^+$ central memory T ($CD4^+$ TCM) cells and $CD8^+$ effector memory T ($CD8^+$ TEM) cells were not included in the HTS analysis.

Since comparative analysis requires accurate normalization, unique molecular identifiers (UMI) were used to process sequencing data. In total, we obtained an average of 5.51 million sequencing reads from 32 samples using the Illumina sequencing platform. On average, 79.15% (range from 69.38 to 88.7%) of these sequence reads were utilized after filtering out low-quality ones. The number of total and unique sequences, total and unique clones and clone types of rearranged TCRVβ products for each sample is listed in **Table S2**. In particular, the number of unique CDR3 sequences, unique CDR3aa and clone types in KO-$CD4^+$ TEM cells showed a downward trend, but no statistical difference was observed compared to WT.

Light Restriction on the TCRVβ Repertoires in $WASp^{-/-}$ $CD4^+$ TEM Cells but not in $WASp^{-/-}$ $CD8^+$ TCM Cells

TCRVβ repertoire diversity and clonality were assessed using several widely used diversity parameters: Shannon–Wiener index, Simpson index, D50, and Chao 1 index. Consistent with our findings in WAS patients, TCR repertoire was selectively skewed in $CD45RO^+CD4^+$ T cells. Compared to WT, KO-$CD4^+$ TEM cells had a higher Simpson index and lower D50 diversity index, indicating an unequal distribution of clonotypes and more clonotypic expansions in KO-$CD4^+$TEM cells. There was no statistical difference in the Shannon index and Chao 1 index, representing the comparable richness and abundance between WT and KO in $CD4^+$ TEM cells (**Figure 1A**). No difference between WT and KO in $CD8^+$ TCM cells for these four parameters was found (**Figure 1B**). In addition, clonal expansion was further assessed by the cumulative frequencies of unique versus total CDR3 clonotypes and the Top 100, which corresponds to the percentage of top 100 CDR3 sequences in the total number of sequences. At the same time, the results showed no marked difference between WT and KO in $CD4^+$ TEM cells or $CD8^+$ TCM cells (**Figures 1C, D**). To further analyze the abundance and sample diversity, we estimated Rank abundance and true diversity (^{q}D) by observing their corresponding curves. The abundance curve of KO-$CD4^+$ TEM cells was steeper and shorter, and ^{q}D curves of KO-$CD4^+$ TEM cells were clearly lower than that of WT, therefore suggesting WASp deficiency decreased the uniformity and diversity of TCRVβ repertoire in $CD4^+$ TEM cells. In comparison, the rank abundance and sample diversity curves of $CD8^+$ TCM cells in WT and KO were almost overlapped (**Figures 1E, F**).

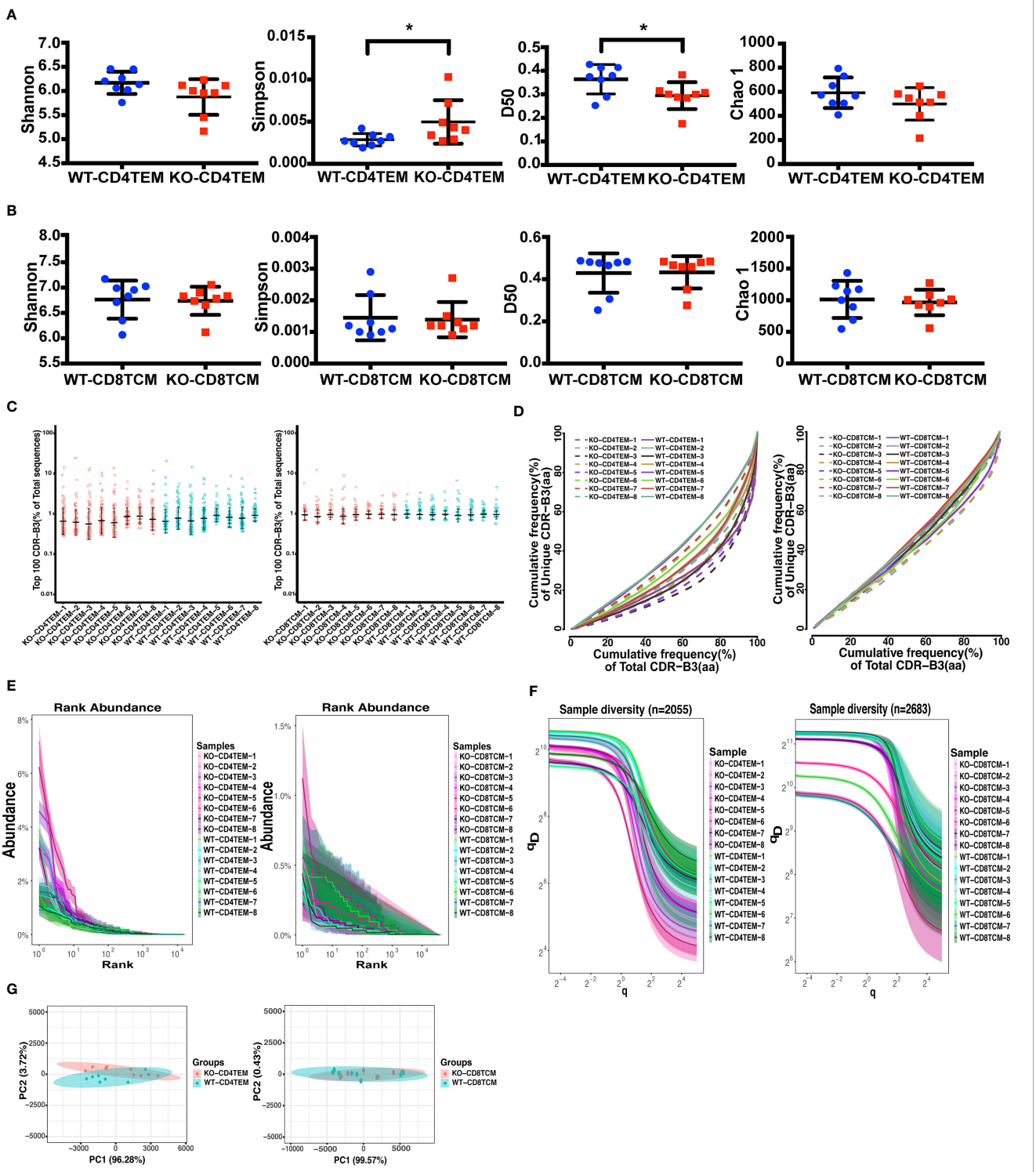

FIGURE 1 | Diversity and clonality analysis of TCRVβ repertoires of CD4$^+$ TEM cells and CD8$^+$ TCM cells in WT and KO chimeras. Quantification of the diversity, unevenness, clonotypic expansion, and richness of TCRVβ repertoires using the Shannon–Wiener index, Simpson index, D50, and Chao 1 index in CD4$^+$ TEM cells **(A)** or CD8$^+$ TCM cells **(B)**. Representation of the frequency of the top 100 most abundant clones for *TRB* sequences **(C)**. The cumulative frequencies of unique versus total CDR3 clonotypes are shown for TCRβ repertoires **(D)**. Mean values ± SE are shown; t-test was used for statistical analysis, *p <0.05. Showing is the Rank-abundance curve and sample diversity curve by using the abundance of total *TRB* sequences versus *TRB* sequences Rank **(E)** and true diversity (^{q}D) versus q **(F)**. Sample plots illustrating the segregation of the various KO from WT chimeras based on primary component (PC) 1 and 2 determined by four variables (Shannon–Wiener index, Simpson, number of total and unique sequences) for TCRVβ repertoires **(G)**.

To assess whether analysis of the TCRVβ repertoire of $CD4^+$ TEM cells and $CD8^+$ TCM cells may distinguish KO chimeras from WT, we used Principal Component Analysis (PCA) based on four variables: Shannon–Wiener index, Simpson, the number of total and unique sequences. As expected, PCA successfully segregated $CD4^+$ TEM cells from WT and KO but failed to discriminate between $CD8^+$ TCM cells from WT and KO (**Figure 1G**). Collectively, WASp-deficiency slightly affects the TCRVβ diversity of $CD4^+$ TEM cells in the chimeric mice model, but not the $CD8^+$ TCM cells.

Skewed Usage of V, D and J Segment Genes in $WASp^{-/-}$ Memory T Cells

The *V(D)J* recombination is the first determinant of TCR diversity. Analysis of *TRB* sequences composition helps understand the usage of individual *V*, *D*, and *J* elements. As shown in the heat map, we analyzed the proportion of *V*, *D* and *J* segment genes among the total sequences of *TRB*. We found no apparent non-stochastic restriction on the usage of *V*, *D* and *J* segments in KO chimeras compared with WT (**Figures 2A, B**). Then, we further compared each *V*, *D* and *J* subfamily genes and found significant differences in the usage of V, D and J segments between $CD4^+$ TEM cells and $CD8^+$ TCM cells in both WT and KO chimeras as expected (**Figures 2C, D**). However, compared to $CD4^+$ TEM cells, the upregulation of TRBV4 and the downregulation of TRBJ1.4 usage in $CD8^+$ TCM cells were found explicitly in KO chimeras. In contrast, the downregulation of TRBV23, TRBJ1.3, and TRBJ1.5 were specifically found in WT chimeras (**Figures 2C, D**). The usage of TRBV12.2 and TRBD1 was upregulated, and that of TRBV30, TRBV31, TRBV4, TRBD2, TRBJ1.1, and TRBJ1.4 was downregulated when comparing $CD4^+$ TEM cells in WT and KO (**Figure 2E**). As for the comparison of $CD8^+$ TCM cells in WT and KO, the usage of TRBV12.2 was increased, TRBV20 was decreased, and D and J segments showed no difference (**Figure 2F**). We also analyzed the composition of the unique sequences of *TRB*, and the results were almost consistent with those in the total sequence (**Figure S2**). Thus, WASp deficiency disturbed the usage of V, D and J genes of $CD4^+$ TEM cells, and V gene segments of $CD8^+$ TCM cells.

Altered Combinations of V(D)J Genes in $WASp^{-/-}$ $CD4^+$ TEM Cells and $WASp^{-/-}$ $CD8^+$ TCM Cells

To further explore whether WASp participates in the combination of V(D)J genes, we analyzed the combination of individual V, D and J genes in total *TRB* sequences. In the combination of V and J genes, the lower right part of KO-$CD4^+$ TEM group was more cluttered compared to the WT group, suggesting that some combination of V–J genes in $CD4^+$ TEM cells was altered in KO chimeras (**Figure 3A**). In contrast, the difference between WT and KO in $CD8^+$ TCM cells was not apparent (**Figure 3B**). To assess overall differences among each individual, we used PCA analysis based on V gene segments, V–J genes combination or V(D)J genes combination to display four compare groups. The pictures showed that V genes and the combination of V–J genes as well as V(D)J genes of $CD4^+$ TEM cells and $CD8^+$ TCM cells could be clearly distinguished into two groups in WT and KO chimeras. In contrast, the group of WT-$CD4^+$ TEM cells was more consistent than KO-$CD4^+$ TEM. WT and KO chimeras are distinguishable from the PCA analysis based on V genes in both $CD4^+$ TEM cells and $CD8^+$ TCM cells. And the PCA analysis based on the combination of V–J genes and V(D)J genes still can be divided into two groups of WT and KO chimeras in both $CD4^+$ TEM cells and $CD8^+$ TCM cells, but less difference was found than that based on V genes (**Figure 3C**). We also showed the analysis of PCA based on the V gene segments and J gene segments. The difference in $CD4^+$TEM cells between WT and KO is attributed more to the selection of TRBV12.2, TRBV30, TRBV31, TRBV4, TRBJ1-1, and TRBJ1-4 genes, while the distinguishable clustering of $CD8^+$TCM cells between KO and WT was more influenced by the selection of TRBV12.2, TRBV15, TRBV20, and TRBV3 genes (**Figure 3D**).

The Higher Similarity of TCRVβ Repertoires of $WASp^{-/-}$ $CD4^+$TEM Cells and $CD8^+$ TCM Cells

The highly shared TCR repertoires are enriched in clonotypes bearing fewer insertions and were reported in autoimmune diseases like type 1 diabetes (24). To detect the degree of sequence sharing, we calculated overlap indices for TCRVβ repertoires of $CD4^+$ TEM cells and $CD8^+$ TCM cells. As presented in the distance heat map, $CD4^+$ TEM cells and $CD8^+$ TCM cells were more similar among KO chimeras than WT. WT and KO were more similar among $CD4^+$ TEM cells than $CD8^+$ TCM cells (**Figure 4A**). The value of overlap indices for $CD4^+$ TEM/$CD4^+$ TEM was lower (**Figure 4B**), while the one for $CD8^+$ TCM/$CD8^+$ TCM was higher in KO than WT (**Figure 4C**). The one for $CD4^+$ TEM/$CD8^+$ TCM in KO was increased compared to WT (**Figure 4D**). In addition, we found a high degree of sharing for TCRVβ sequences between $CD4^+$ TEM and $CD8^+$ TCM in KO chimeras. To assess the relative similarity of TCRVβ repertoires in different ways, we further used the VDJTOOLS software to visualize repertoire overlaps of 'F2', 'R', and 'D' metrics. Metric F2 reflects the relative share occupied by the common clonotypes in two groups; Metric R is the overall similarity of repertoire organization; Metric D ignores clonotype frequencies and reflects the number of shared clonotypes between the two groups (25). We found that R and D metrics of $CD4^+$ TEM cells and $CD8^+$ TCM cells in KO were more diffuse, suggesting higher similarity of shared sequences. F2, R, and D metrics of KO-$CD8^+$ TCM samples were more aggregated than WT, indicating different shared clonotypes between the two groups (**Figure 4E**).

Differences in the Amino Acid Composition of TCRVβ Repertoires Caused by WASp Deficiency

To assess the global amino acid composition of TCRVβ repertoires, we used a biological parameter, Bhattacharyya distance, to analyze the similarity between samples at the

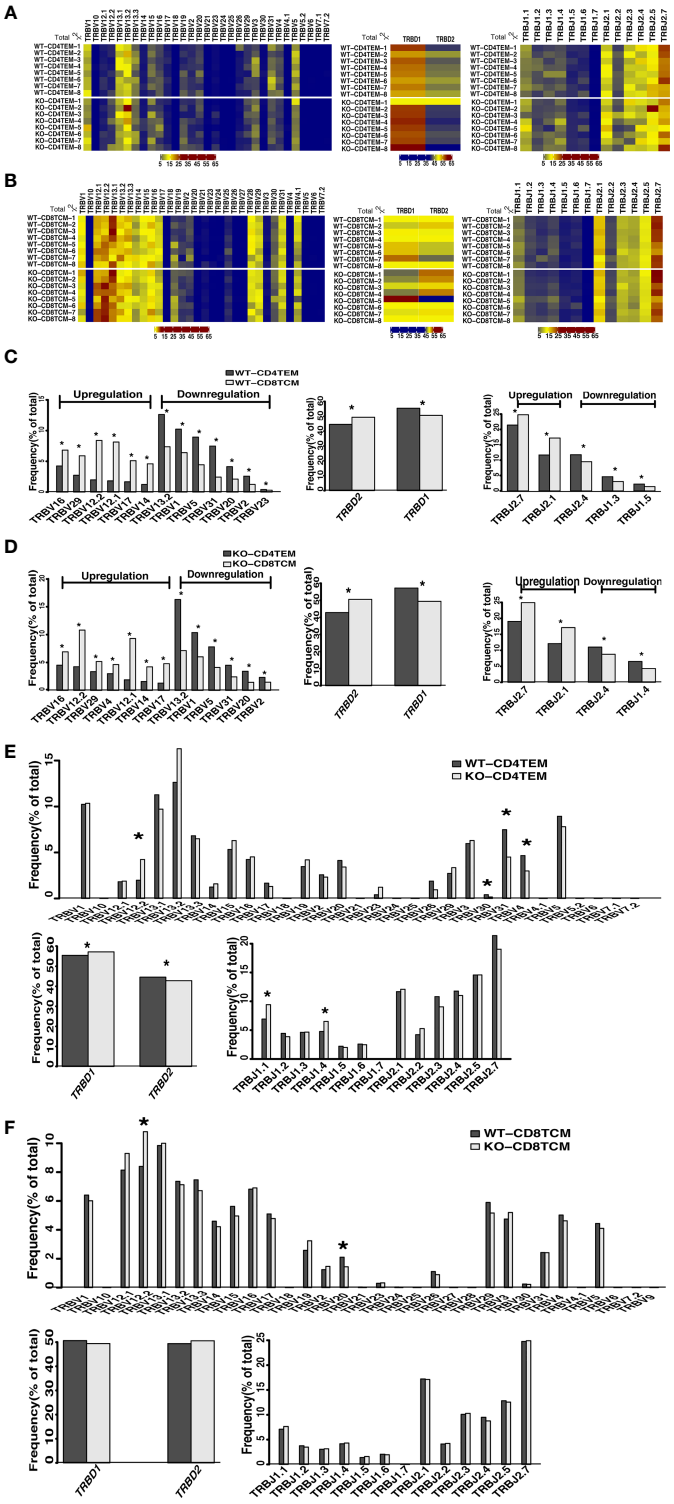

FIGURE 2 | Differential usage of V, D and J genes in the total sequences of *TRB* repertoires of CD4$^+$ TEM cells and CD8$^+$ TCM cells in WT and KO chimeras. Heatmap represents V, D and J gene usage frequency for total *TRB* sequences of CD4$^+$ TEM cells **(A)** and CD8$^+$ TCM cells **(B)** in WT and KO chimeras. Relative frequency for the usage of TRBV, TRBD, and TRBJ gene segments for CD4$^+$ TEM cells vs CD8$^+$ TCM cells in WT chimeras **(C)**, CD4$^+$ TEM cells vs CD8$^+$ TCM cells in KO chimeras **(D)**, WT vs KO chimeras in CD4$^+$ TEM cells **(E)** and WT vs KO chimeras in CD8$^+$ TCM cells **(F)**. *$p < 0.05$.

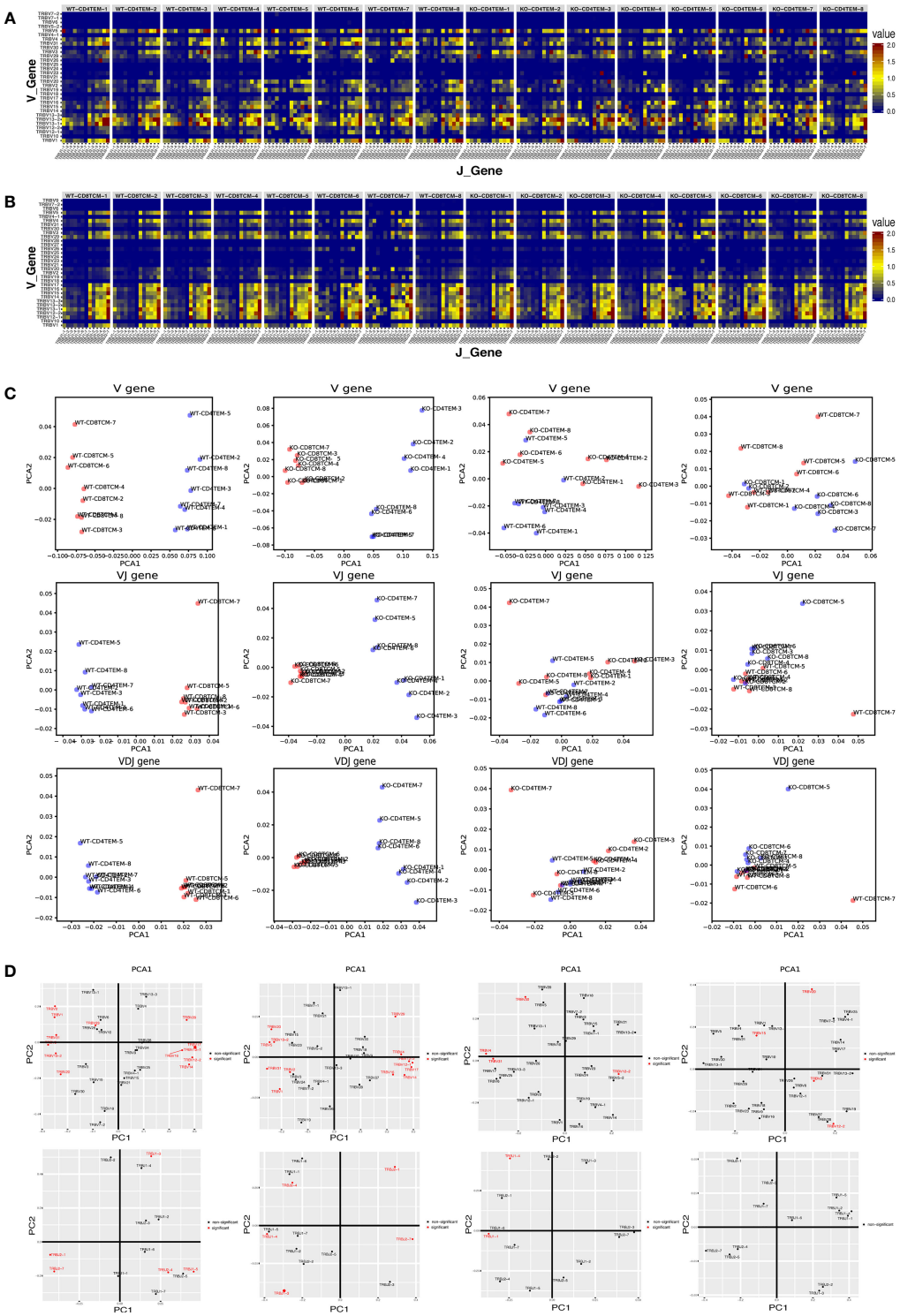

FIGURE 3 | Differential V–J and V–D–J combination of CD4$^+$ TEM cells and CD8$^+$ TCM cells in WT and KO chimeras. Heatmap representing the frequency of V–J combination for CD4$^+$ TEM cells **(A)** and CD8$^+$ TCM cells **(B)** in WT and KO chimeras. Sample plots illustrating the segregation of CD4$^+$ TEM cells from CD8$^+$ TCM cells in WT chimeras, CD4$^+$ TEM cells from CD8$^+$ TCM cells in KO chimeras, KO from WT chimeras in CD4$^+$ TEM cells, and that of KO from WT chimeras in CD8$^+$ TCM cells (from left to right) based on PCA of V genes, the combination of V–J and also V–D–J, distribution of V gene families and J gene families (from up to down) **(C)**. V and J gene families with significant differences were shown in red **(D)** ($p < 0.05$).

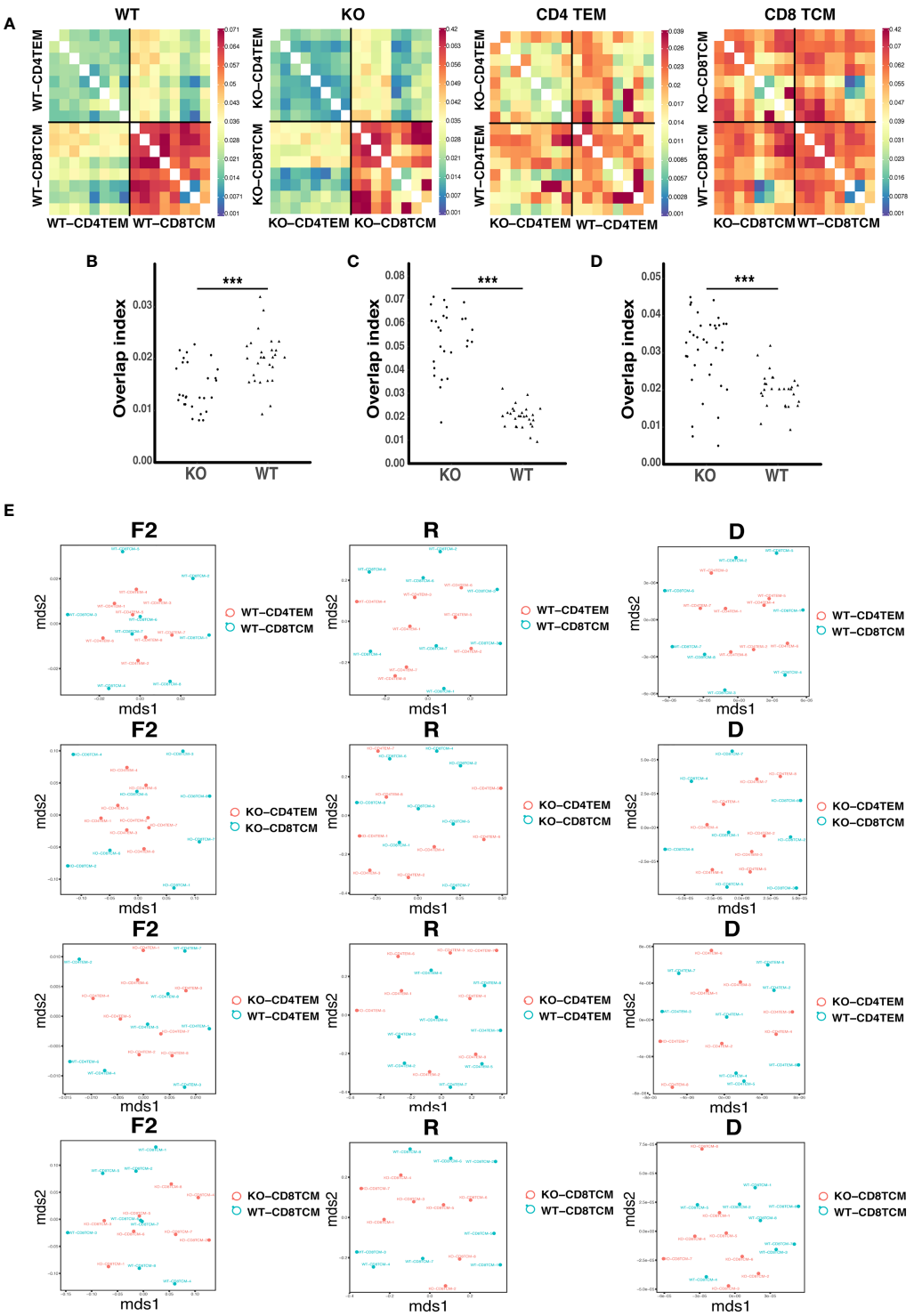

FIGURE 4 | The relative similarity of TCRVβ repertoires in WT and KO chimeric mice. Heatmap represents the distance matrix of TCRVβ repertoires of $CD4^+$ TEM and $CD8^+$ TCM cells in WT and KO chimeric mice **(A)**. Overlap indices for $CD4^+$ TEM/$CD4^+$ TEM **(B)**, $CD8^+$ TCM/$CD8^+$ TCM **(C)** and $CD4^+$ TEM/$CD8^+$ TCM **(D)**. Metrics F2, R and D for $CD4^+$ TEM and CD8 TCM TRB sequences in WT and KO chimeric mice **(E)**. ***$p < 0.001$.

amino acid level. We found that there was no difference in all of the four compare groups: WT-CD4$^+$TEM vs WT-CD8$^+$TCM, KO-CD4$^+$TEM vs KO-CD8$^+$TCM, KO-CD4$^+$TEM vs WT-CD4$^+$TEM, and KO-CD8$^+$TCM vs WT-CD8$^+$TCM (**Figure 5A**). Then, we analyzed the usage of each amino acid and found parts of amino acid usage were significantly different among those four groups (**Figure 5B**). When CD8$^+$TCM cells were compared with CD4$^+$TEM cells, downregulation of Glutamine (Q), Methionine (M) usage and upregulation of Valine (V), Proline (P) usage was specifically found in WT, while downregulation of Aspartic acid (D) and upregulation of Tryptophan (W) usage was specifically found in KO chimeras. In CD4$^+$ TEM cells, the usage of Tyrosine (Y) was decreased, and the usage of Phenylalanine (F), Asparagine (N) and V was increased in KO compared to WT. D usage was lower and W was higher when KO-CD8$^+$TCM cells were compared with WT (**Figure 5C**).

WASp Deficiency Did Not Affect the Hydrophobicity and the Length of TCRVβ Sequences

Differences in the compositions of amino acids may change the hydrophilicity and hydrophobicity of TCR. The hydrophobicity of TCR and the length of TCRVβ sequences are related to autoimmune diseases, as previously reported (22, 27). About 24 to 72% of WAS patients have autoimmune diseases (28). Therefore, we analyzed the hydrophobicity of amino acids at positions 6 and 7 as reported. And no significant difference was found between KO and WT groups in CD4$^+$ TEM cells and CD8$^+$ TCM cells (**Figures 6A, B**). A previous study (27) has shown that cysteine and hydrophobic residues in CDR3 serve as distinct T-cell self-reactivity indices. We further calculated the hydrophobic index and cysteine index, and found no significant difference in KO-CD4$^+$TEM vs WT-CD4$^+$TEM, and KO-CD8$^+$TCM vs WT-CD8$^+$TCM (**Figures 6C, D**). The results suggest that WASp deficiency did not obviously alter stochastic process of TCR assembly to produce more cysteine and hydrophobic residues in CD4$^+$ TEM cells and CD8$^+$ TCM cells in relatively young mice. Furthermore, the length of CDR3β nucleotide among the total sequences and unique sequences showed a negative difference again (**Figure 7A**). We then calculated the complexity score and skewness index of total and unique sequences, and they still showed no changes in CD4$^+$ TEM cells and CD8$^+$ TCM cells between KO and WT (**Figures 7B, C**).

DISCUSSION

TCR diversity is an essential guarantee for effective T cell immunity. Previous studies have confirmed that TCR diversity can be affected by many factors such as age, pathogen infection, tumor, autoimmune diseases, immunization, and immunosuppression (18, 19). Limitations to diversity may be a feature of V(D)J rearrangement that is as significant to immune function as the bewildering number of lymphocyte specificities that can theoretically be generated. As proved by other researchers and our team, TCR diversity was severely impaired, mainly in the memory T cell populations in WAS patients (16, 17). However, whether the TCR diversity limitation in WAS was caused by intrinsic WASp deficiency is still unclear. In 2014, Petersen et al. showed that TCR diversity was limited in old WAS$^{-/-}$ mice, but not in young ones. They only detected the total T cells, ignoring that WASp deficiency could selectively affect the diversity of T cell subsets (20). In this study, for the first time, we used WAS chimeric mice model to study the TCR diversity in WAS, which could exclude the potential influence of other WASp deficient immunocytes and other affecting factors. Our work revealed that the limited TCRVβ diversity of CD4$^+$ TEM cells and CD8$^+$ TCM cells in WAS are intrinsic but not severe. Moreover, WASp-deficiency affected the TCR diversity of CD4$^+$ TEM cells more than CD8$^+$ TCM cells, indicating WASp may play a more critical role in forming TCR diversity of CD4$^+$ TEM cells than that of CD8$^+$ TCM cells.

The mechanisms for the limitations to TCR diversity of CD4$^+$ TEM cells and CD8$^+$ TCM cells in WAS are still unknown. WASp is involved in the process of T cell maturation, differentiation, and proliferation (29, 30). WASp-deficiency affects the maturation and differentiation of T cells inside and outside the thymus. Studies have shown that T cell lymphopenia was common in young WAS patients (31). As there is no limited TCR diversity of naïve T cells in young WAS patients (16), and Petersen et al. had shown that TCR diversity in thymus and spleen was not limited in young WASp$^{-/-}$ mice (20), we also found no limited TCR diversity of naïve CD4 and CD8 T cells in nonchimeric WASp$^{-/-}$ mice (data unpublished), so the impaired TCR diversity of memory T cells may not be related to the deficiency of thymus output. Since WASp-deficiency results in impaired T cell survival and abnormal memory formation efficiency, TCR repertoire analysis of different memory T cells in WAS could provide additional clues regarding the biophysical properties of the TCRs of CD4$^+$ TEM cells that may potentially affect the process of memory T cell formation. Due to the limited number of cells, the TCR diversity of CD4$^+$ TCM cells and CD8$^+$ TEM cells was not studied in this study. Whether the limited TCR diversity of CD4$^+$ TEM cells was a continuation of CD4$^+$ TCM cells is unknown. CD4$^+$ TEM are more susceptible to the effects of WASp deficiency, since its constitutive generation across mouse life likely through TCR driven events. In contrast, CD8$^+$ TCM in mice are largely generated in earlier life with a less antigen driven pathway. Even if the mechanisms of their development are not fully understood, it appears to be cytokine-dependent (32). This may explain why few perturbations to TCR repertoire diversity are found in CD8$^+$ TCM. Additionally, whether the slightly skewed TCR diversity of CD8$^+$ TCM cells could lead to impaired TCR diversity of CD8$^+$ TEM cells also remain to be further studied. All in all, the specific mechanism of TCR diversity restriction of memory T cells caused by WASp-deficiency has yet to be further defined.

Comparing V- and J-segment usage frequencies may reflect the functional differences in TCR repertoires and the biases in thymic recombination machinery. This study showed that WASp-

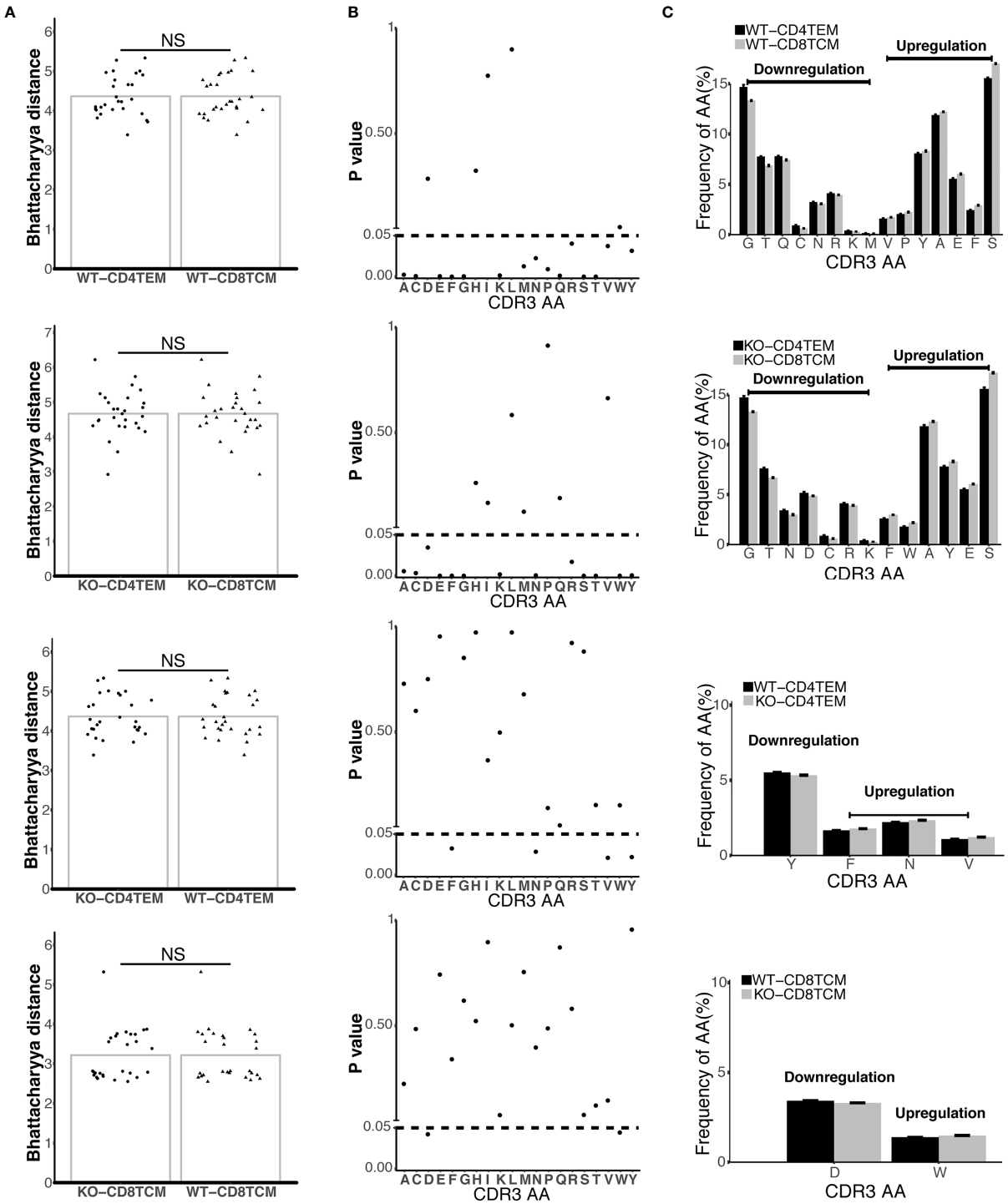

FIGURE 5 | Differential composition of CDR3 amino acids (aa) in TCRVβ repertoires from four compare groups. Bhattacharyya distance analysis for the similarity of CDR3aa from four compare groups: $CD4^+$ TEM vs. $CD8^+$ TCM in WT chimeras, $CD4^+$ TEM vs. $CD8^+$ TCM in KO chimeras, WT vs. KO chimeras in $CD4^+$ TEM, and WT vs. KO chimeras in $CD8^+$ TCM (from up to down) **(A)**. The frequencies of 20 aa in CDR3 from four compare groups **(B)**. Downregulation and upregulation of CDR3 AA in the different groups were shown **(C)**. $p < 0.05$. NS, No Significant.

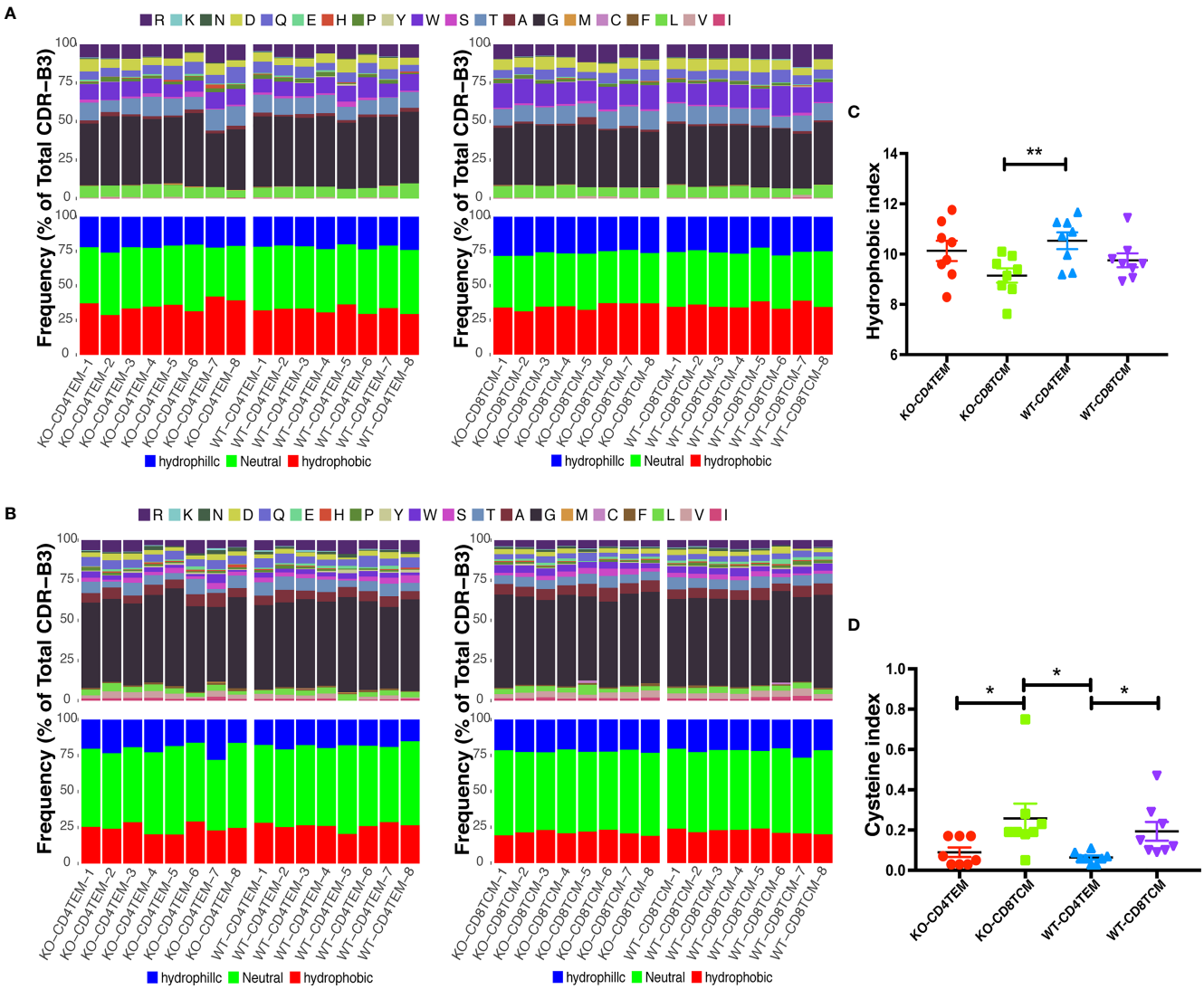

FIGURE 6 | Hydrophobicity of amino acids (aa) in CDR3β repertoires of CD4$^+$ TEM cells or CD8$^+$ TCM cells from WT and KO chimeras. Composition of aa residues at positions 6 **(A)** and 7 **(B)** of the 13 aa-long CDR3β. Hydrophobic index **(C)** and cysteine index **(D)** in CDR3β repertoires. *p < 0.05; **p < 0.01.

deficiency affected the usage of V, D, and J segment genes, which was consistent with previous studies in WAS patients (17). However, we did not find specific V, D, and J segment genes, fixed upregulation, nor downregulation of V(D)J usage in both WAS patients and mice models. So, the difference in the usage of V, D, and J genes caused by WASp-deficiency may randomly happen. Furthermore, the difference in the usage of V, D, and J segments caused by WASp-deficiency was gradually decreased with the combination of V(D)J. Whether the different usage of V, D, and J genes in WAS was related to specific pathogens' susceptibility or autoimmune diseases still needs more research.

Epidemiological studies showed that 24–72% of patients with WAS had autoimmune diseases, namely, autoimmune hemolytic anemia (AIHA), vasculitis, arthritis, nephropathy, inflammatory bowel disease, and immune granulocytic (28). As reported in type 1 diabetes (24), the highly shared TCR repertoires were enriched in clonotypes with fewer insertions. Our results showed higher sharing of TCRVβ sequences between CD4$^+$ TEM and CD8$^+$ TCM cells in WAS chimeric mice than in WT, suggesting that WAS chimeric mice are more prone to autoimmunity than WT. However, the segments associated with autoimmune diseases, like TRBV2, TRBV6, and TRBV8.2, were not upregulated in WAS chimeric mice. Since we found no direct association of autoimmunity and V(D)J gene levels, we further detected the hydrophobicity of amino acids at positions 6 and 7, and the length of TCRVβ sequences in amino acid levels. The differences in the compositions of amino acids may change the hydrophilicity and hydrophobicity of TCR, and a previous study showed that the interfacial hydrophobicity of amino acids at positions 6 and 7 of the CDR3β segment robustly promotes the development of self-reactive TCRs (33). Also, the

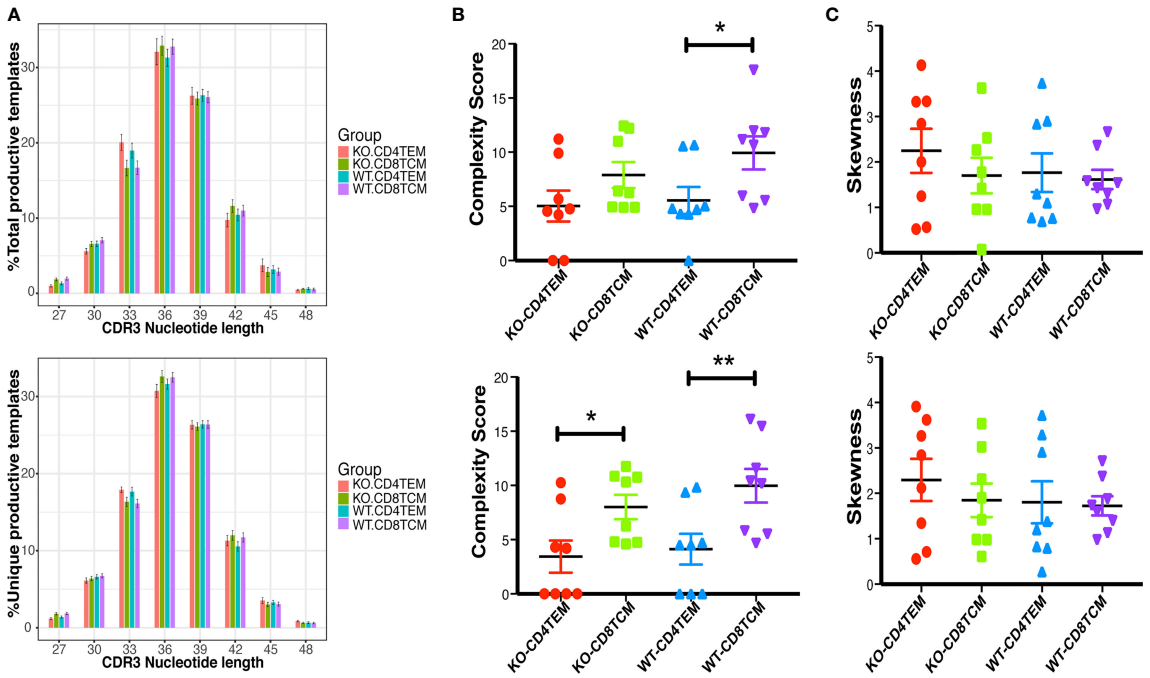

FIGURE 7 | Distribution of the length of CDR3β nucleotide of CD4$^+$ TEM cells or CD8$^+$ TCM cells from WT and KO chimeras. Distribution of the length of the CDR3β region of TRB total sequences (up) and unique sequences (down) **(A)**. Complexity scores **(B)** and skewness **(C)** of CDR3β total sequences (up) and unique sequences (down). *p < 0.05, **p < 0.01.

length of TCRVβ sequences is related to autoimmune diseases (22, 27). As a result, although we found differences in the composition of amino acid of TCRVβ repertoires between WAS chimeric mice and the WT, no significant difference in amino acid hydrophobicity at positions 6 and 7 was found. A previous study showed that (27), an increased cysteine index was a specific biomarker of defective cortical tolerance mechanisms, the hydrophobic index appeared more sensitive for detection of a self-tolerance defect but was not specific for either cortical or medullary tolerance mechanisms. Thus the cysteine and hydrophobic indices provide complementary information in the diagnosis and classification of T-cell self-tolerance defects. Therefore, we detected both indices in each cell subpopulation, but there were no significant difference between KO and WT in both CD4$^+$ TEM and CD8$^+$ TCM cells. This suggested that the WASp defect did not affect the self-tolerance of CD4$^+$ TEM and CD8$^+$ TCM in thymus. O'Connell et al. reported a change in the length of the TCR sequence in WAS patients (17). However, our data showed no significant change in the length of TCRVβ sequences between WAS chimeric mice and the WT. All these data in our study were insufficient to prove a direct association between the alteration of WAS TCR diversity and autoimmunity. This may be due to an early investigation before the onset of autoimmune disease, which is more often seen in old WASp$^{-/-}$ mice.

The quality of comparative repertoire analysis relies on the TCR library preparation, sequencing (TCR-seq) methods and the following software analysis algorithms (25). This study used 5' RACE-PCR, deep sequencing, and UMI quantification to the maximum extent to remove technical bias. However, these techniques and methods are still expected to be further optimized. In addition, compared to human samples, the mouse model has similar genetic homogeneity and strengthened repertoire convergence. Therefore, even limited available T cell counts often create the possibility of clear and statistically significant results concerning the characteristics and similarity of syngeneic mouse TCR repertoires for the different T cell subsets, different age groups, and in various transgenic mouse models (25). Although we used chimeric mice to exclude those possible interference factors, there may still be differences between mice and humans, and our findings in mice need further validation in younger WAS patients without infections.

Overall, we confirmed that the effect of WASp-deficiency on the TCRVβ diversity of CD4$^+$ TEM cells and CD8$^+$ TCM cells was not severe but intrinsic. The intrinsically disturbed TCRVβ diversity in WAS chimeric mice provided clues for researchers to explore the mechanism of autoimmunity and infection in WAS patients. These results also help further study the function of WASp and the specific mechanism of WASp affecting TCR diversity.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI bioproject repository, accession number PRJNA792306.

(accessible at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA792306).

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Usage Committee of Children's Hospital of Chongqing Medical University.

AUTHOR CONTRIBUTIONS

WL performed this research, analyzed data, and wrote the paper. YJ, YW, QZ, LY, TZ, LN, and RD provided help in performing the research. YL reviewed and revised the manuscript. XZ and JW designed the study, reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.794795/full#supplementary-material

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Phenomic Analysis of Chronic Granulomatous Disease Reveals More Severe Integumentary Infections in X-Linked Compared With Autosomal Recessive Chronic Granulomatous Disease

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Background: Chronic granulomatous disease (CGD) is an inborn error of immunity (IEI), characterised by recurrent bacterial and fungal infections. It is inherited either in an X-linked (XL) or autosomal recessive (AR) mode. Phenome refers to the entire set of phenotypes expressed, and its study allows us to generate new knowledge of the disease. The objective of the study is to reveal the phenomic differences between XL and AR-CGD by using Human Phenotype Ontology (HPO) terms.

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Methods: We collected data on 117 patients with genetically diagnosed CGD from Asia and Africa referred to the Asian Primary Immunodeficiency Network (APID network). Only 90 patients with sufficient clinical information were included for phenomic analysis. We used HPO terms to describe all phenotypes manifested in the patients.

Results: XL-CGD patients had a lower age of onset, referral, clinical diagnosis, and genetic diagnosis compared with AR-CGD patients. The integument and central nervous system were more frequently affected in XL-CGD patients. Regarding HPO terms, perianal abscess, cutaneous abscess, and elevated hepatic transaminase were correlated with XL-CGD. A higher percentage of XL-CGD patients presented with BCGitis/BCGosis as their first manifestation. Among our CGD patients, lung was the most frequently infected organ, with gastrointestinal system and skin ranking second and third, respectively. *Aspergillus* species, *Mycobacterium bovis*, and *Mycobacteirum tuberculosis* were the most frequent pathogens to be found.

Conclusion: Phenomic analysis confirmed that XL-CGD patients have more recurrent and aggressive infections compared with AR-CGD patients. Various phenotypic differences listed out can be used as clinical handles to distinguish XL or AR-CGD based on clinical features.

Keywords: chronic granulomatous disease (CGD), inborn error of immunity (IEI), human phenotype ontology (HPO), phenome, genetics

INTRODUCTION

Chronic granulomatous disease (CGD) is an inborn error of immunity (IEI) that is characterised by recurrent infections caused by catalase-positive bacteria and fungi, such as *Staphylococcus aureus* and *Aspergillus* species (1). It is estimated that the prevalence of CGD is 1 in 250,000 live births among Europeans and Americans (2, 3). CGD arises from the loss of function of one of the proteins forming the NADPH oxidase complex, which generates reactive oxygen species, i.e., superoxide radicals and hydrogen peroxide for intracellular bacteria and fungi killing in phagocytes (4). Currently, there is one X-linked (XL) and five autosomal recessive (AR) forms of CGD. The gene responsible for XL-CGD is *CYBB*, and the other five genes responsible for AR-CGD are *CYBA*, *NCF1*, *NCF2*, *NCF4*, and *CYBC1*. Frequently affected organs and systems include the lung, skin, lymph node, and liver (3). Patients may suffer from pneumonia and deep and superficial abscesses. CGD patients usually present with lymphadenopathy and hepatosplenomegaly on physical examination (5).

In our study, we focus on the phenomic analysis of CGD. Phenomics stands for the acquisition of high-dimensional phenotypic data on an organism scale. Study of phenomics is usually incorporated with the study of genomics and environment so that we can know various factors which might possibly influence the complex traits displayed. Compared with genomics, phenomics is much more sophisticated and is much more difficult to be characterised (6). For this study, we use Human Phenotype Ontology (HPO) terms to describe the phenomic abnormalities. The HPO project was publicised in 2008 and provides an ontology of annotations (7), i.e., HPO terms, to describe phenotypic abnormalities encountered by clinicians. The HPO currently contains over 13,000 terms arranged in a simple class-subclass relationship such that various specific terms belong to a subclass of a parent term. The aim of the HPO system is to offer a computational bridge between genome biology and clinical medicine, as well as enabling the integration of phenotypic information across various scientific tools for clinical diagnosis and research purposes (7). Due to advancement in technology, there are more external tools available for genomic discovery project and diagnostic research. For genomic projects, HPO terms are used to filter out the list of candidate genes to be tested from whole genome sequencing using Exomiser, Phevor, or PhenIX (8, 9). External algorithms such as Phenomizer and Phenolyzer can compute clinical phenotype data written in HPO terms to give out possible differential diagnoses. Phenomizer is an external tool which utilises HPO terms to report phenotypic abnormalities. It yields a list of differential diagnoses of the patient based on the HPO data inputted by a clinician (10). However, for most IEI which are included in the genotypic classification of the International Union of the Immunological Societies, a full HPO phenomic data is still lacking currently which requires contributions from different clinical immunologists (11, 12). Therefore, it is paramount to generate a phenome of IEI for a reference for the differential diagnosis tool. This can provide sufficient information for them to diagnose future patients by importing HPO terms identified by medical practitioners.

Currently, numerous case series about CGD patients have already been published but none of them has used HPO terms to represent their phenome. The phenomic data on CGD patients stored in Phenomizer database may not be accurate as well due to

insufficient phenomic analysis. As a result, the main aim of this project is to observe and create a phenome for CGD patients in our case series. We also attempted to identify the main differences of the phenotypic data between XL-CGD and AR-CGD patients. The phenome of the respective XL or AR-CGD patients may also be sent to various external tools which use HPO terms for analysis such as Phenomizer and Exomiser so as to provide a reference for diagnosis and genetic analysis of suspected CGD patients. Significant differences are listed out to help clinicians to differentiate between them clinically.

MATERIALS AND METHODS

Patient Source and Selection

The APID network is an IEI referral network established in 2009 by The University of Hong Kong, which acts as a platform to offer e-consultation and free genetic testing for IEI. There are over 100 member centres across Asia and Africa. From 2003 to 2017, 117 CGD patients referred from 18 centres were successfully genetically diagnosed and were included in this case series.

Data Collection

Clinical records and laboratory results of CGD patients, provided by their referring doctors at the time of request for genetic testing, are archived in the APID network. Patient data, including demographics, family history, age of clinical milestones, infection, and genetic results were recorded.

Phenomic Data

HPO (October 2020 version) terms, which describe individual phenotypic abnormalities in a hierarchical framework of organs, were applied for performing the phenomic analysis. Two researchers first reviewed the laboratory reports and clinical notes and then suitable HPO terms were selected from the HPO browser http://www.human-phenotype-ontology.org to describe the phenotypic abnormalities displayed. In the end, a HPO phenotypic profile for each CGD patient was generated. The HPO phenotypic profile consisting of all HPO terms which can be manifested from the clinical records was selected. No negated HPO terms, i.e., no specific phenotype was manifested in the clinical record, were used in our study. Discrepancies for the final HPO phenotypic profile were discussed and modified. Only the highest class of HPO terms in the hierarchial framework, i.e., systems affected and the most specific HPO terms were computed for detection of any significant correlation between individual phenotypic abnormality and mode of inheritance.

Genetics Data

Genetic analysis was performed using genomic DNA extracted from peripheral blood. Genomic DNA was sent to our research laboratory and the candidate genes, i.e., *CYBB*, *CYBA*, *NCF1*, *NCF2*, and *NCF4*, were tested by using Sanger sequencing on the basis of clinical likelihood in the research laboratory of the Department of Paediatrics and Adolescent Medicine, The University of Hong Kong. Pathogenicity of the targeted gene mutation is re-evaluated in accordance with the diagnostic interpretation guidelines published by the American Academy of Allergy, Asthma & Immunology (AAAAI) PID working group in 2020 (13).

APID Network Questionnaire

A questionnaire was also distributed online to APID network member centres across Asia and Africa. Questions regarding the availability of care for CGD patients in APID network member centres, i.e., diagnosis, laboratory tests, and treatment of CGD patients were asked. A total of 20 responses were recorded.

Ethics Approval

This research project is approved by The University of Hong Kong/Hospital Authority Hong Kong West Cluster Institutional Review Board.

Statistical Analysis

For descriptive statistics, all ages of clinical milestones were expressed in median and range (year). Univariate analysis was performed by independent-samples Mann-Whitney *U* test to evaluate the difference between XL-CGD and AR-CGD. First manifestation, HPO terms, and system affected are presented in the form of heat map and expressed in percentages. Fisher's exact test was used in analysis to determine the correlation between categorical phenotypes with the genotype.

RESULTS

Demographics Data

The demographics data of our CGD case series was displayed in the Sankey diagram in **Figure 1**. Of the 117 patients, 104 (88.9%) were male and 13 (11.1%) were female. XL-CGD was seen in 87 (74.4%) patients, while the remaining belonged to AR-CGD group. Out of the 30 AR CGD patients, 9 (30.0%) of them were found to have mutations in *CYBA* by Sanger Sequencing, 13 (43.3%) of them were found to have GT deletion in *NCF1* by GeneScan® and 9 (30.0%) of them were found to have mutations in *NCF2* by Sanger Sequencing, with one of them have concurrent *CYBA* and *NCF2* mutations diagnosed. In our case series, more than half of the patients (56.7%) came from mainland China, with India (17.3%) and Hong Kong (17.3%) ranking second. Other patients either came from South-east Asia or South Africa. Details about consanguinity and family history were only available for 92 out of 117 patients, with 90 (97.8%) having no family consanguinity and 2 (2.2%) with family consanguinity. Moreover, out of the 92 patients, 69 (75.0%) have no suggestive family history and 23 (25.0%) have suggestive family history. Only 1 of them has an elder brother with known CGD.

Genetics Data

The genetic mutations of 117 CGD patients, with 3 patients reporting 2 unphased variants, are shown in **Table 1**. The commonest gene implicated was *CYBB*, with no diagnosed *NCF4*

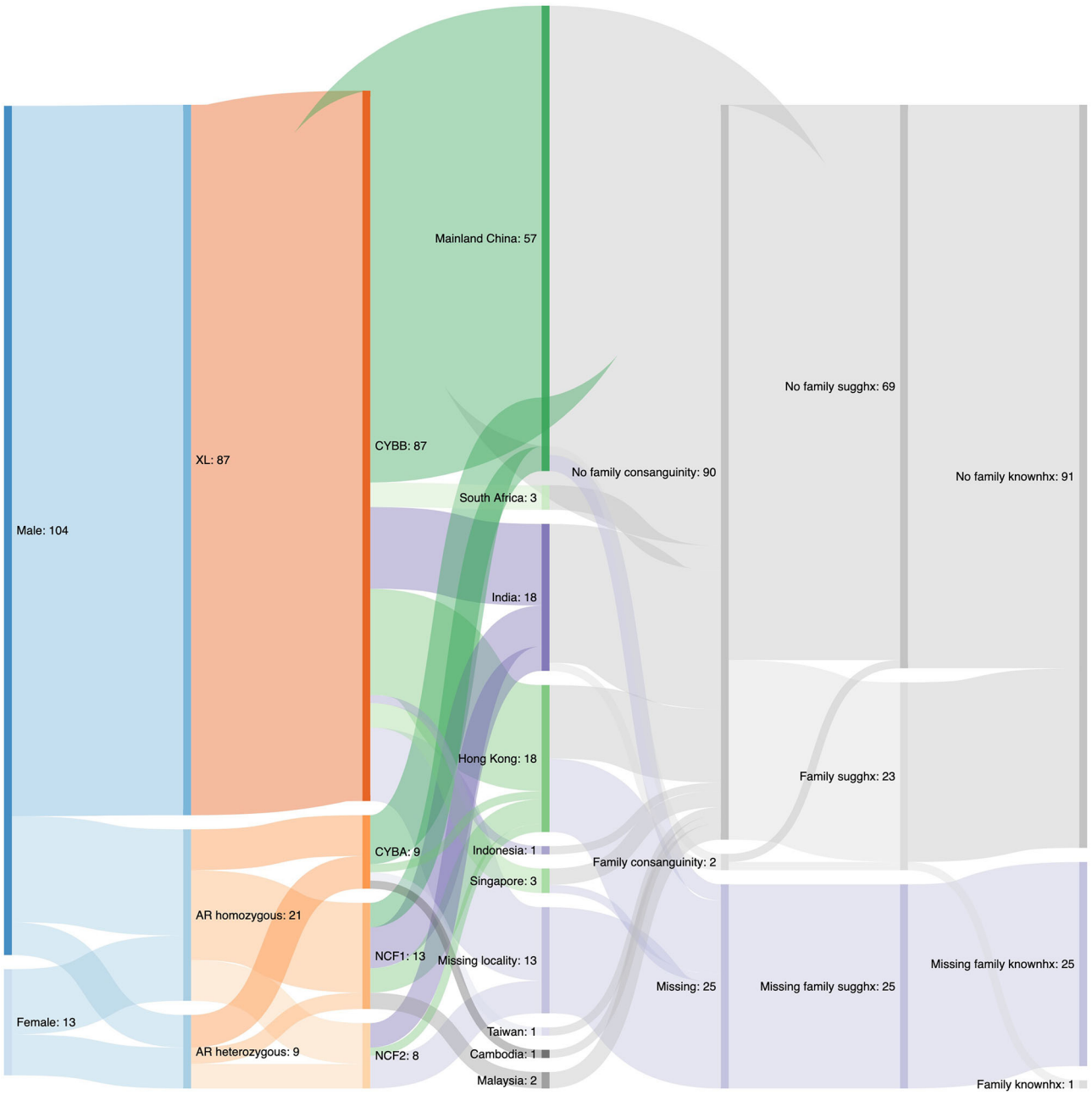

FIGURE 1 | A Sankey diagram describing demographics data of the CGD case series. XL, X-linked; AR, autosomal recessive; sugghx, suggested history; knownhx, known history.

CGD patients. In total, 87 of them had mutations identified in *CYBB* gene. For the remaining 30 AR-CGD patients, 20 of them had homozygous mutations while only 1 of them was confirmed with compound heterozygous. Four of them had 2 unphased heterozygous mutations found in a recessive gene and five of them had 1 heterozygous mutation found in a recessive gene.

All AR-CGD patients with *NCF1* mutations have documented GT deletion. Four patients among our CGD case series had a large deletion mutation in *CYBB*. There were 24 novel mutations identified in our patients, including 23 mutations in *CYBB* and 1 mutation in *NCF2*. Pathogenicity of these unreported mutations was determined by using the AAAAI guidelines in 2020 (13). Among 117 CGD patients, 99 of the CGD patients have pathogenic variants, 13 of them have likely pathogenic variants, and 5 of them have variants with uncertain significance.

TABLE 1 | Genetics data of CGD patients.

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
1	M	CYBB	X-linked hemizygous	LRG_53t1:c.252G>A	p.Ala84=, predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PS3)**	(14), ClinVar: VCV000010933.6
2	M	CYBB	X-linked hemizygous	LRG_53t1:c.252G>A	p.Ala84=, predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PS3)**	(14), ClinVar: VCV000010933.6
3	M	CYBB	X-linked hemizygous	LRG_53t1:c.742dup	p.Ile248Asnfs*36	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(14)
4	M	CYBB	X-linked hemizygous	LRG_53t1:c.613T>A	p.Phe205Ile	Missense	**Likely pathogenic (PM2, PM1, PP4')**	(15, 16),
5	M	CYBB	X-linked hemizygous	LRG_53t1:c.1498G>C	p.Asp500His	Missense	**Pathogenic (PS3, PM1, PM2, PP4')**	(14)
6	M	CYBB	X-linked hemizygous	LRG_53t1:c.1555G>T	p.Glu519*	Nonsense	**Pathogenic (PVS1, PS2, PM2, PP4')**	(15, 16),
7	M	CYBB	X-linked hemizygous	LRG_53t1: c.646_648del	p.Phe216del	Deletion	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(17)
8	M	CYBB	X-linked hemizygous	LRG_53t1:c.1025T>A	p.Leu342Gln	Missense	**Likely pathogenic (PM2, PP4', PP5, PP2)**	(16)
9	M	CYBB	X-linked hemizygous	LRG_53t1:c.713del	p.Val238Glyfs*4	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(16, 18),
10	M	CYBB	X-linked hemizygous	LRG_53t1:c.1327del	p.Trp443Glyfs*59	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(16, 18),
11	M	CYBB	X-linked hemizygous	LRG_53t1:c.935T>A	p.Met312Lys	Missense	**Likely pathogenic (PM2, PM1, PP4',)**	(16, 18),
12	M	CYBB	X-linked hemizygous	LRG_53t1:c.1437C>A	p.Tyr479*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(16, 18),
13	M	CYBB	X-linked hemizygous	LRG_53t1:c.253-1G>A (RT-PCR: LRG_53t1: c.253_266del)	RT-PCR show aberrant splicing, Cys85Serfs*13	Splice site	**Pathogenic (PVS1, PM2, PP4')**	(11)
14	M	CYBB	X-linked hemizygous	LRG_53t1:c.46-1G>C	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
15	M	CYBB	X-linked hemizygous	LRG_53t1:c.577T>C	p.Ser193Pro	Missense	**Pathogenic (PS3, PM2, PM1, PP4')**	(14, 18),
16	M	CYBB	X-linked hemizygous	LRG_53t1:c.868C>T	p.Arg290*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(14, 18),
17	M	CYBB	X-linked hemizygous	LRG_53t1:c.1315-2A>C	/	Splice site	**Pathogenic (PVS1, PM2, PP4')**	(16, 19),
18	M	CYBB	X-linked hemizygous	LRG_53t1:c.1713A>T	p.*571Tyrext*8	Elongation	**Likely pathogenic (PM2, PP4', PM1)**	Not reported
19	M	CYBB	X-linked hemizygous	LRG_53t1:c.77_78del	p.Phe26Cysfs*8	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(7)
20	M	CYBB	X-linked hemizygous	LRG_53t1:c.469C>T	p.Arg157*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(20, 21),
21	M	CYBB	X-linked hemizygous	LRG_53t1: c.857_867del	p.Val286Alafs*58	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	Not reported
22	M	CYBB	X-linked hemizygous	LRG_53t1:c.676C>T	p.Arg226*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(19), ClinVar: VCV000010929.5
23	M	CYBB	X-linked hemizygous	LRG_53t1:c.742del	p.Ile248Serfs*7	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(22)
24	M	CYBB	X-linked hemizygous	LRG_53t1:c.1234G>A	p.Gly412Arg	Missense	**Likely pathogenic (PM2, PP4', PM1)**	Not reported

(Continued)

TABLE 1 | Continued

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
25	M	CYBB	X-linked hemizygous	LRG_53t1:c.674 +608_1587-1407del (EX7-EX11del)	p.Arg226Profs*14	Large deletion	**Pathogenic (PVS1, PM2, PP4')**	Not reported
26	M	CYBB	X-linked hemizygous	LRG_53t1:c.804 +2T>C	Predicted aberrant splicing	Splicing	**Pathogenic (PVS1, PM2, PP4')**	(16, 21),
27	M	CYBB	X-linked hemizygous	LRG_53t1:c.1583C>G	p.Pro528Arg	Missense	**Likely pathogenic (PM2, PP4', PM1)**	Not reported
28	M	CYBB	X-linked hemizygous	LRG_53t1:c.626A>G	p.His209Arg	Missense	**Pathogenic (PS3, PM2, PP4', PM1)**	(23)
29	M	CYBB	X-linked hemizygous	LRG_53t1:c.45+1G>A	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	(24)
30	M	CYBB	X-linked hemizygous	LRG_53t1: c.1164_1166delinsATC	p.Asp388_Gly389delinsGluSer	In-frame deletion/ insertion	**Likely pathogenic (PM2, PP4', PM5)**	Not reported
31	M	CYBB	X-linked hemizygous	LRG_53t1:c.1014C>A	p.His338Gln	Missense	**Likely pathogenic (PM2, PP4', PM1)**	(16, 25),
32	M	CYBB	X-linked hemizygous	LRG_53t1:c.1399G>T	p.Glu467*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(16, 22),
33	M	CYBB	X-linked hemizygous	LRG_53t1:c.1016C>A	p.Pro339His	Missense	**Pathogenic (PS3, PM2, PP4', PM1)**	(17)
34	M	CYBB	X-linked hemizygous	EX11-EX13del	Predicted no protein expression	Large deletion	**Pathogenic (PVS1, PM2, PP4')**	Not reported
35	M	CYBB	X-linked hemizygous	LRG_53t1:c.911C>G; EX11-EX13del	p.Pro304Arg and predicted protein truncation	Missense and large deletion	**Pathogenic (PVS1, PM2, PP4')**	(25, 26),
36	M	CYBB	X-linked hemizygous	LRG_53t1:c.925G>A	p.Glu309Lys	Missense	**Pathogenic (PS3, PM2, PP4', PM1)**	(17)
37	M	CYBB	X-linked hemizygous	LRG_53t1: c.1150_1151 +2delAAGT	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	(27)
38	M	CYBB	X-linked hemizygous	LRG_53t1:c.376T>C	p.Cys126Arg	Missense	**Pathogenic (PS2, PS3, PM2, PP4', PM1)**	(25)
39	M	CYBB	X-linked hemizygous	LRG_53t1:c.1332del	p.Cys445Alafs*57	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	Not reported
40	M	CYBB	X-linked hemizygous	LRG_53t1:c.469C>T	p.Arg157*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(20, 21),
41	M	CYBB	X-linked hemizygous	LRG_53t1:c.70_72del	p.Phe24del	Deletion	**Likely pathogenic (PM2, PP4', PM1)**	(22)
42	M	CYBB	X-linked hemizygous	LRG_53t1:c.676C>T	p.Arg226*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(19), ClinVar: VCV000010929.5
43	M	CYBB	X-linked hemizygous	LRG_53t1:c.665A>G	p.His222Arg	Missense	**Pathogenic (PS3, PM2, PP4', PM1)**	(19)
44	M	CYBB	X-linked hemizygous	LRG_53t1:c.1244C>T	p.Pro415Leu	Missense	**Likely pathogenic (PM2, PP4', PM1)**	(19),
45	M	CYBB	X-linked hemizygous	LRG_53t1:c.1313del	p.Lys438Argfs*64	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(16, 21),
46	M	CYBB	X-linked hemizygous	LRG_53t1:c.1328G>A	p.Trp443*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(16, 19),
47	M	CYBB	X-linked hemizygous	LRG_53t1: c.126_130delinsTTTC	p.Arg43Phefs*18	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(16)
48	M	CYBB	X-linked hemizygous	LRG_53t1:c.868C>T	p.Arg290*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(18, 28),
49	M	CYBB	X-linked hemizygous	LRG_53t1:c.674 +6T>C	Predicted aberrant splicing	Splicing	**Pathogenic (PVS1, PM2, PP4')**	Not reported
50	M	CYBB	X-linked hemizygous	LRG_53t1: c.1619_1626dup	p.Ala543Lysfs*7	Frameshift with	**Pathogenic (PVS1, PM2, PP4')**	Not reported

(Continued)

TABLE 1 | Continued

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
51	M	CYBB	X-linked hemizygous	LRG_53t1:c.1038del	p.Glu347Argfs*39	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	Not reported
52	M	CYBB	X-linked hemizygous	LRG_53t1:c.141+3A>T	Predicted aberrant splicing	Splicing	**Pathogenic (PVS1, PM2, PP4')**	Not reported
53	M	CYBB	X-linked hemizygous	LRG_53t1:c.271C>T	p.Arg91*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(21, 29),
54	M	CYBB	X-linked hemizygous	LRG_53t1:c.1151 +1G>A	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
55	M	CYBB	X-linked hemizygous	LRG_53t1:c.1314 +2T>G	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
56	M	CYBB	X-linked hemizygous	LRG_53t1:c.45+1G>A	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
57	M	CYBB	X-linked hemizygous	LRG_53t1:c.469C>T	p.Arg157*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(21)
58	M	CYBB	X-linked hemizygous	LRG_53t1:c.742dup	p.Ile248Asnfs*36	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(21)
59	M	CYBB	X-linked hemizygous	LRG_53t1:c.1548G>C	p.Trp516Cys	Missense	**Likely pathogenic (PM2, PP4', PP5, PP2)**	(16)
60	M	CYBB	X-linked hemizygous	LRG_53t1:c.252G>A	p.A84=	Splice site	**Pathogenic (PVS1, PS3)**	(30), ClinVar: VCV000010933.6
61	M	CYBB	X-linked hemizygous	LRG_53t1:c.123C>G	p.Tyr41*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	Not reported
62	M	CYBB	X-linked hemizygous	LRG_53t1:c.676C>T	p.Arg226*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(19), ClinVar: VCV000010929.5
63	M	CYBB	X-linked hemizygous	LRG_53t1:c.868C>T	p.Arg290*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(18, 29),
64	M	CYBB	X-linked hemizygous	LRG_53t1:c.675-1G>T	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
65	M	CYBB	X-linked hemizygous	LRG_53t1:c.-65C>T	/	Promoter	**Likely pathogenic (PS3, PM2, PP4')**	(31)
66	M	CYBB	X-linked hemizygous	LRG_53t1:c.1022C>T	p.Thr341Ile	Missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(16, 32),
67	M	CYBB	X-linked hemizygous	LRG_53t1:c.742dup	p.Ile248Asnfs*36	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(14, 21),
68	M	CYBB	X-linked hemizygous	LRG_53t1:c.252G>A	p.A84=	Splice site	**Pathogenic (PVS1, PS3)**	(30), ClinVar: VCV000010933.6
69	M	CYBB	X-linked hemizygous	LRG_53t1: c.724_725del	p.Thr242Serfs*3	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(33)
70	M	CYBB	X-linked hemizygous	Exon 8-13 deletion	/	Large deletion	**Pathogenic (PVS1, PM2, PP4')**	Not reported
71	M	CYBB	X-linked hemizygous	LRG_53t1: c.714_715insTA	p.His239Tyrfs*4	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(34)
72	M	CYBB	X-linked hemizygous	LRG_53t1:c.45+1G>C	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
73	M	CYBB	X-linked hemizygous	LRG_53t1:c.84G>A	p.Trp28*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(35)
74	M	CYBB	X-linked hemizygous	LRG_53t1:c.1154T>G	p.Ile385Arg	missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(16, 32),
75	M	CYBB	X-linked hemizygous	LRG_53t1:c.1075G>A	p.Gly359Arg	missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(14, 32),

(Continued)

TABLE 1 | Continued

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
76	M	CYBB	X-linked hemizygous	LRG_53t1:c.217C>T	p.Arg73*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(35)
77	M	CYBB	X-linked hemizygous	LRG_53t1: c.1322_1324del	p.Phe441del	In-frame deletion/ insertion	**Uncertain significance (PM2, PP4')**	Not reported
78	M	CYBB	X-linked hemizygous	LRG_53t1:c.141 +1_141+2del	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
79	M	CYBB	X-linked hemizygous	LRG_53t1:c.1546T>C	p.Trp516Arg	Missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(36)
80	M	CYBB	X-linked hemizygous	LRG_53t1:c.676C>T	p.Arg226*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(19), ClinVar: VCV000010929.5
81	M	CYBB	X-linked hemizygous	LRG_53t1:c.676C>T	p.Arg226*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(19), ClinVar: VCV000010929.5
82	M	CYBB	X-linked hemizygous	LRG_53t1: c.722_726delTAACA	p.Ile241Serfs*3	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(19)
83	M	CYBB	X-linked hemizygous	LRG_53t1:c.388C>T	p.Arg130*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(21)
84	M	CYBB	X-linked hemizygous	LRG_53t1:c.45+2delT	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
85	M	CYBB	X-linked hemizygous	LRG_53t1:c.1414G>A	p.Gly472Ser	Missense	**Likely pathogenic (PM2, PP4', PP5, PP2)**	(25)
86	M	CYBB	X-linked hemizygous	LRG_53t1:c.985T>C	p.Cys329Arg	Missense	**Pathogenic (PM2, PP4', PP5, PP2)**	(37)
87	M	CYBB	X-linked hemizygous	LRG_53t1:c.868C>T	p.Arg290*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(18)
88	F	CYBA	Compound heterozygous	LRG_52t1:c.70G>A	p.Gly24Arg	Missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(38–40)
				LRG_52t1:c.204-2A>G	predicted aberrant splicing	splice site	**Pathogenic (PVS1, PM2, PP4')**	
89	M	CYBA	Heterozygous	LRG_52t1:c.418G>A	p.Glu140Lys	Missense	**Uncertain significance (PM2, PP4')**	ClinVar: VCV000966844.1
90	F	CYBA	2 heterozygous (not known if compound heterozygous)	LRG_52t1:c.7C>T	p.Gln3*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(40, 41)
				LRG_52t1:c.59-2A>G	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	
91	M	CYBA	Homozygous	LRG_52t1:c.7C>T	p.Gln3*	nonsense	**Pathogenic (PVS1, PM2, PP4')**	(40, 41)
92	F	CYBA	2 heterozygous (not known if compound heterozygous)	LRG_52t1:c.7C>T	p.Gln3*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(40, 41)
				LRG_52t1:c.129-23_129-5del	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	
93	F	CYBA	Homozygous	LRG_52t1:c.7C>T	p.Gln3*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(40, 41)
94	F	CYBA	Homozygous	LRG_52t1: c.482_498delinsC	p.Glu162Leufs*3	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(41)
95	F	CYBA	Homozygous	LRG_52t1:c.371C>T	p.Ala124Val	Missense	**Pathogenic (PS3, PM2, PP4', PP5, PP2)**	(40)
96	M	CYBA	Homozygous	LRG_52t1:c.205G>T	p.Gly69*	Nonsense	**Pathogenic (PVS1, PM2, PP4')**	(42–45), ClinVar: VCV000002248.10
		NCF2	Heterozygous	LRG_88t1:c.1183C>T	p.Arg395Trp	Missense	**Uncertain significance due to conflicting interpretations@**	
97	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with	**Pathogenic (PVS1, PM2, PP4')**	(46)

(Continued)

TABLE 1 | Continued

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
						premature stop codon		
98	F	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
99	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
100	F	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
101	F	NCF1	Heterozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
102	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
103	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
104	F	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
105	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
106	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
107	M	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
108	F	NCF1	Homozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
109	F	NCF1	Heterozygous	LRG_87t1:c.75_76del	p.Tyr26Hisfs*26	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(46)
110	M	NCF2	Homozygous	LRG_88t1: c.835_836del	p.Thr279Glyfs*16	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(30, 47–49)
111	M	NCF2	2 heterozygous (not known if compound heterozygous)	LRG_88t1:c.1099C>T	p.Gln367*	Frameshift with premature stop codon	**Pathogenic (PVS1, PM2, PP4')**	(16, 48)
				LRG_88t1:c.1179-2A>T	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	
112	M	NCF2	Homozygous	LRG_88t1: c.835_836del	p.Thr279Glyfs*16	Frameshift with	**Pathogenic (PVS1, PM2, PP4')**	(30, 47–49)

(Continued)

TABLE 1 | Continued

Patient no	Sex	Gene	Mode of inheritance	cDNA position	Codon change	Mutation type	Pathogenicity#	References
						premature stop codon		
113	M	NCF2	Heterozygous	LRG_88t1:c.1183C>T	p.Arg395Trp	Missense	**Uncertain significance due to conflicting interpretations@**	(43–45), ClinVar: VCV000002248.10
114	M	NCF2	Heterozygous	LRG_88t1:c.1183C>T	p.Arg395Trp	Missense	**Uncertain significance due to conflicting interpretations@**	(43–45), ClinVar: VCV000002248.10
115	F	NCF2	Homozygous	LRG_88t1: c.835_836del	p.Thr279Glyfs*16	Frameshift with premature stop codon	Pathogenic (PVS1, PM2, PP4')	(49)
116	M	NCF2	Homozygous	LRG_88t1:c.501 +1_501+8del	Predicted aberrant splicing	Splice site	**Pathogenic (PVS1, PM2, PP4')**	Not reported
117	M	NCF2	Homozygous	LRG_88t1: c.835_836del	p.Thr279Glyfs*16	Frameshift with premature stop codon	Pathogenic (PVS1, PM2, PP4')	(49)

Mutation nomenclature is made according to Locus Reference Genome (LRG). Mutation pathogenicity curation is made according to the AAAAI guideline 2020.
**PP4' denotes an increase of pathogenicity to "moderate" level, as suggested by the AAAAI PID Genetics guidance.*
Criteria for classifying pathogenic variants as abbreviated by the ACMG Standards and Guidelines for the Interpretation of Sequence Variants: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4544753/.
It is considered pathogenic allele in NCBI SNP database with conflicting interpretation in pathogenicity (ClinVar) in 2017.

Clinical Characteristics of XL and AR-CGD

The ages of clinical milestones of our CGD patients are displayed in **Table 2**. Ages of onset, referral, clinical diagnosis, and genetic diagnosis correlated with the mode of inheritance. Median age of onset correlated with the mode of inheritance ($p = 0.01$), with XL-CGD (0.2 years) lower than that of AR-CGD (0.4 years). Median age of referral to an immunology unit of XL-CGD (0.8 years) is also significantly lower than that of AR-CGD (3.5 years) and was shown to be significantly related with the mode of inheritance ($p = 0.009$). Median age at clinical diagnosis of XL-CGD (1.4 years) is younger than that of AR-CGD (4.8 years) with a strong correlation between XL or AR ($p = 0.017$). The same result was also demonstrated for the age of genetic diagnosis ($p = 0.004$) with XL-CGD patients showing a lower median age (2.2 years) compared with AR-CGD patients (4.8 years).

First manifestations of XL and AR-CGD patients in our case series were displayed in **Figure 2**. Only the 5 most common first manifestations were included in the heat map, namely fever, BCGitis/BCGosis, pneumonia, cough, and lymphadenopathy, where generalized lymphadenopathy and cervical lymphadenopathy or no specific location regarding the lymphadenopathy were all categorized here. As shown in the figure, there is no significant association between the mode of inheritance and the respective first manifestation. However, it could be seen that a higher percentage of XL-CGD (18%) has BCGitis/BCGosis as their first manifestation compared with AR-CGD (4%) while a higher proportion of AR CGD (22%) has lymphadenopathy as their first manifestation compared with XL CGD (10%).

Infection Profile

Results for microbiological testing ordered based on clinical suspicion of infections were tallied, and cultured microorganisms from various infections and the 3 top locations where they were cultured are reported in **Figure 3**. However, the infectious etiology may not be established in every CGD case as the culture of the pathogen may not be performed or the culture reports were negative

TABLE 2 | Comparison between clinical characteristics between XL-CGD and AR-CGD using Fisher's exact test.

Parameter	XL (range) ($n = 67$)	AR (range) ($n = 23$)	p-value
Median age of onset	0.2 (0–5) ($n = 66$)	0.4 (0–13.3) ($n = 19$)	0.01**
Median age of referral to immunology centre	0.75 (0–14) ($n = 64$)	3.5 (0.1–26.6) ($n = 18$)	0.009**
Median age at clinical diagnosis	1.4 (0–14) ($n = 58$)	4.8 (0.2–26.7) ($n = 13$)	0.017
Median age at genetic diagnosis	2.2 (0.1–14.8) ($n = 70$)	4.7 (0.9–26.7) ($n = 19$)	0.004
Median delay in referral to immunology centre	0.3 (0–12) ($n = 59$)	0.25 (0–12) ($n = 14$)	0.794
Median delay in diagnosis of CGD	0 (–0.3–6.2) ($n = 53$)	0.3 (0–1) ($n = 8$)	0.375

*XL, X-linked; AR, autosomal recessive; CGD, chronic granulomatous disease. **$p < 0.01$.*

[First manifestation (in percentages)]

First manifestation	XL total (n=67)	AR total (n=23)	p value
BCGitis/BCGosis HP:0020086/HP:0020087	18	4	0.171
lymphadenopathy HP:0002716	10	22	0.1757
cough HP:0012735	13	17	0.7327
fever HP:0001945	30	30	1
pneumonia HP:0002090	15	13	1

FIGURE 2 | A heat map describing percentages of CGD patients in our case series with certain first manifestations according to their clinical records. XL, X-linked; AR, autosomal recessive; BCG, Bacillus Calmette–Guérin. Fisher's exact test is used, *p*-value <0.05 is significant. This graph is created by using the app Datawrapper.

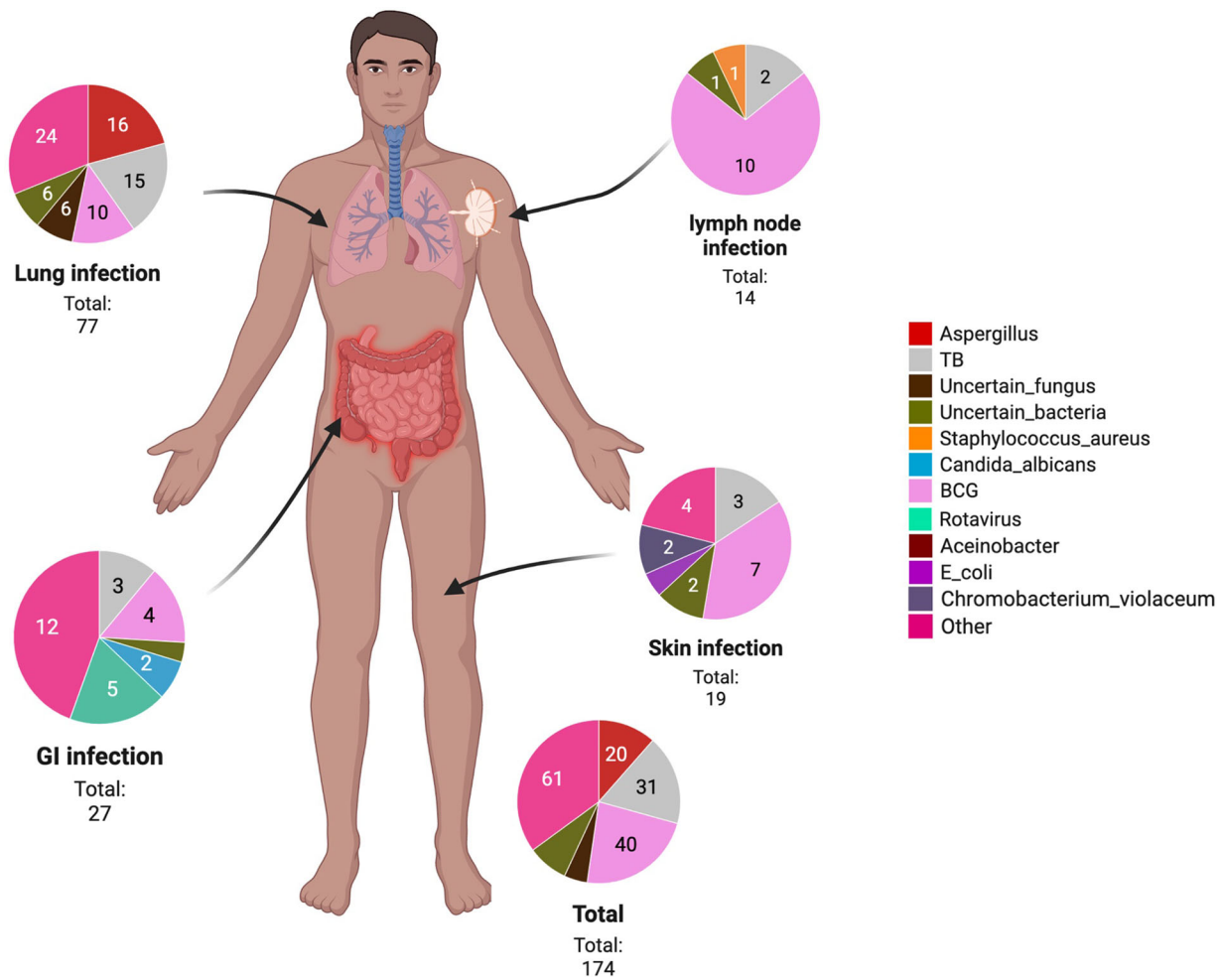

FIGURE 3 | A body map showing the infection location of pathogens in our CGD case series. GI, gastrointestinal; TB, *Mycobacterium tuberculosis*; *E. coli*, *Escherichia coli*; BCG, Bacillus Calmette–Guérin.

due to the use of antibiotics or antifungals prior to the culture. As shown above, a significant portion of infection was caused by uncertain bacteria or fungi. In general, the most common pathogens isolated are the *Mycobacteirum bovis* (n = 40), *Mycobacteirum tuberculosis* (n = 31), and *Aspergillus* species (n = 23). *M. bovis* was mostly isolated in the lungs, lymph nodes and the skin. *M. tuberculosis* was isolated in multiple systems or organs including the lungs, gastrointestinal system, skin, or disseminated infection while *Aspergillus* species were cultured mostly in the lungs. The most frequent locations of infections are the lungs (n = 77), gastrointestinal system (n = 27), and the skin (n = 19). Other locations of infection include the heart (n = 1), upper respiratory tract (n = 1), central nervous system (n = 1), ear (n = 2), neck (n = 2), spleen (n = 6), liver (n = 3), lymph node (n = 14), bone (n = 7), and disseminated infection (n = 3). Among the 77 episodes of infection affecting the lungs, *Aspergillus* species (n = 18) and *M. tuberculosis* (n = 15) were most often isolated, causing mostly pneumonia and bronchitis. Among the 23 episodes of infection affecting the gastrointestinal system, *Rotavirus* (n = 5) was most often isolated from the alimentary canal, causing mostly diarrhoea, vomiting, and enterocolitis. Other infectious etiologies causing hepato-splenomegaly and hepatic and splenic abscesses were not included here. Among the 19 episodes of infections affecting the skin, *M. bovis* (n = 7) is the most commonly isolated, causing cutaneous abscesses most of the time.

Phenomic Analysis Between XL and AR-CGD

Regarding the phenomic analysis, we only included 90 out of 117 patients as only these 90 patients had sufficient clinical information provided. The affected systems of XL and AR-CGD patients are displayed in **Figure 4** in the form of a heat map. In general, more XL-CGD patients are affected compared with AR-CGD patients in terms of the systems. Immune system was not shown in the heat map because all CGD patients had their immune system affected. As shown above, the most frequently affected systems of both XL and AR-CGD patients were the respiratory system, homeostasis system, and digestive system, respectively. A univariate analysis was performed to determine the correlation between various systems and their respective genotypes by using Fisher's exact test. The integumentary system is significantly associated between the mode of inheritance (p = 0.0153), with more XL-CGD patients (57%) affected compared with AR-CGD patients (26%). In addition, more XL-CGD patients (13%) have their nervous system affected compared with no AR-CGD patients showing

[HPO system heat map (in percentages)]

System	XL total (n=67)	AR total (n=23)	p value
Integument HP:0001574	57	26	0.0153**
Nervous system HP:0000707	13	0	0.1047
Growth HP:0001507	19	9	0.3374
Respiratory system HP:0002086	81	70	0.3828
Homeostasis HP:0001939	79	70	0.3964
Musculoskeletal system HP:0033127	27	17	0.4153
Cardiovascular system HP:0001626	16	9	0.5024
Digestive system HP:0025031	78	70	0.5743
Genitourinary system HP:0000119	7	4	1.0000
Ear HP:0000598	7	9	1.0000
Head or neck HP:0000152	12	13	1.0000

FIGURE 4 | A heat map describing percentages of CGD patients in our case series in which their organ systems are affected. HPO, Human Phenotype Ontology. **$p < 0.01$. This graph is created by using the app Datawrapper.

such manifestation. Although it is not statistically significant, it is still an interesting phenomenon to be reported.

Phenotypic abnormalities of CGD patients are displayed in **Figure 5** in the form of a heat map as shown above. More than 200 HPO terms describing phenotypic abnormalities were recorded in our CGD case series but only HPO terms which were manifested by more than 10% of patients would be included in the heat map. In general, more HPO terms were displayed in XL-CGD patients compared with AR-CGD patients. Among all the HPO terms recorded, both recurrent fever and pneumonia are the most frequent HPO terms identified with more than 70% of XL-CGD patients and 50% of AR-CGD patients showing this phenotypic abnormality. Other more common phenotypic abnormalities include hepatosplenomegaly, cutaneous abscess, and anaemia.

A total of 5 HPO terms were shown to be correlated with XL or AR inheritance by Fisher's exact test with more XL-CGD patients showing that specific phenotypic abnormality. Perianal abscess, cutaneous abscess and elevated hepatic transaminase are strongly correlated with the mode of inheritance ($p < 0.001$). Bronchitis and cough are correlated with the mode of inheritance as well ($p < 0.05$).

APID Network Questionnaire Regarding the Care of CGD Patients

The results of the questionnaire which was delivered to 20 APID network members are shown in **Figure 6**. As displayed, there were 16 centres who had diagnosed CGD in their clinics and most of them diagnosed their first CGD patients in the 1990s to 2010s. Only 9 APID network members have performed nitroblue tetrazolium test (NBT) after their establishment. APID network members diagnosed CGD by using NBT test initially with all of the 9 clinics performing the first NBT test before 2010s. However, starting from 1990s, APID network members started to use dihydrorhodamine (DHR) cytometry assay as well. In total, 11 APID network members have performed DHR cytometry assay in their clinics during the 1990s to 2010s.

DISCUSSION

Our study revealed that XL-CGD and AR-CGD patients had some phenotypic differences through phenomic analysis. XL-CGD patients had their integument and the central nervous system more frequently affected. XL-CGD patients were shown to have perianal abscess, cutaneous abscess, and elevated hepatic transaminase more often as well. More XL-CGD patients presented with BCGitis/BCGosis as their first manifestation.

In our study, the most significant finding is that the integument system is more frequently affected among XL-CGD patients than AR-CGD patients. The reason behind such finding is due to more frequent perianal abscess, perianal rash, and cutaneous abscess reported among XL-CGD patients in the phenomic analysis, as it was observed in previous publications (3, 50–52). However, from reports in India and China, there is no statistical difference between XL and AR-CGD patients with episodes of superficial abscess (30, 41). Another interesting

[Common HPO terms heat map (in percentage)]

Common HPO terms	XL total (n=67)	AR total (n=23)	p value
Perianal abscess HP:00097898	33	4	0.0056**
Cutaneous abscess HP:0031292	45	13	0.0063**
Elevated hepatic transaminase HP:0002910	22	0	0.0096**
Bronchitis HP:0012387	27	4	0.0349*
Cough HP:0012735	46	22	0.0492*
Perianal rash HP:0011131	24	4	0.0606
Granuloma HP:0032252	15	35	0.0666
Recurrent fever HP:0001954	75	52	0.0666
Neutrophilia in the presence of infection HP:0410257	27	9	0.086
Splenomegaly	48	26	0.0887
Increased circulating IgA level HP:0003261	19	39	0.0894
Sepsis HP:0100806	33	13	0.1041
BCGitis HP:0020086	36	17	0.1223
Anemia HP:0001903	45	26	0.1426
Skin ulcer HP:0200042	10	0	0.1841
Pneumonia HP:0002090	73	57	0.1914
Increased circulating IgM level HP:0003496	18	30	0.2405
BCGosis HP:0020087	16	4	0.2836
Anal fistula HP:0010447	7	0	0.3231
Productive cough HP:0031245	7	0	0.3231
Increased circulating IgG level HP:0003237	36	48	0.3312
Extrapulmonary TB HP:0032271	9	0	0.3318
Tonsillitis HP:0011110	9	0	0.3318
Pyoderma HP:0000999	19	9	0.3374
FTT secondary to recurrent infections HP:0008866	19	9	0.3374
Thrombocytosis HP:0001894	24	13	0.3791
Vomit HP:0002013	7	13	0.4162
Recurrent pneumonia HP:0006532	33	22	0.4312
Cervical lymphadenopathy HP:0025289	34	43	0.4603
Hepatomegaly HP:0002240	45	35	0.4689
Pulmonary TB HP:0032262	12	17	0.4945
Otitis media HP:0000388	4	9	0.5989
Cellulitis HP:0100658	6	9	0.6432
Mediastinal lymphadenopathy HP:0100721	9	4	0.673
Enterocolitis HP:0004387	9	4	0.673
Recurrent URTI and LRTI HP:0200117	10	4	0.6743
Osteomyelitis HP:0002754	10	4	0.6743
Oral ulcer HP:0000155	10	4	0.6743
Thrombocytopenia HP:0001873	9	13	0.6885
Generalized lymphadenopathy HP:0008940	18	13	0.7515
Diarrhoea HP:0002014	31	35	0.7991
Increased circulating total IgE level HP:0003212	12	9	1
Splenic abscess HP:0025059	15	13	1
Bloody diarrhoea HP:0025085	13	13	1
Hepatic abscess HP:0100523	7	4	1

FIGURE 5 | A heat map describing percentages of CGD patients in our case series where their clinical records displayed certain phenotypic abnormalities. Fisher's exact test is used for statistical analysis (Only more than 5% of patients describing certain HPO term is recorded). HPO, Human Phenotype Ontology. Note: $^{**}p < 0.01$; $^{*}p < 0.05$. This graph is created by using the app Datawrapper.

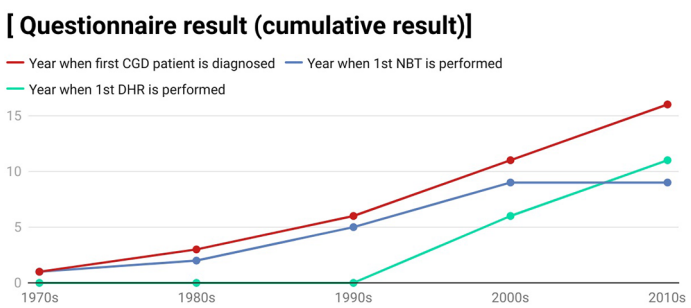

FIGURE 6 | A line graph showing the questionnaire result which we deliver to various immunology centres. This graph is created by using the app Datawrapper.

TABLE 3 | A summary of findings regarding the differences between previous case series and this case series.

CGD case series (year published)	Region	Total no of patients (XL/AR)	Percentage of XL patients	Percentage of male patients	Median or mean age of onset in years (XL/AR)	Median or mean age of diagnosis in years (XL/AR)	Most common infectious etiology	Most common infection location	Frequency of BCGitis (XL/AR)	Mortality rate	First manifestation
This case series	Asia and Africa	118	75%	89%	(0.2/0.4)	(1.4/4.8)	*M. bovis*	Lungs	(36%/17%)	/	fever
Rawat et al. (30)	India	236	44%	73%	0.7 (0.5/1.0)	2.0 (1.0/2.5)	*Aspergillus*	Lungs	/	60%	/
Blancas-Galicia et al. (57)	Mexico	93	77%	88%	0.3	2.5	*S. aureus*	Lungs	58%	40%	/
Gao et al. (41)	China	159	89%	89%	/	1.3	*M. tuberculosis* and *Aspergillus*	Lungs	/	43%	/
Zhou et al. (22)	China	169	89%	89%	0.1 (0.1/0.1)	0.7 (0.7/0.8)	*S. aureus*	Lungs	59%	37%	recurrent fever
Oliveira-Junior et al. (58)	Latin America	71	75%	82%	2.0 (1.8/2.8)	4.4 (3.6/8.2)	*S. aureus*	Lungs	30%	0%	/
Bortoletto et al. (52)	USA	27	70%	85%	/	3 (2.1/5.3)	*S. aureus*	Lungs	/	15%	/
Rawat et al. (29)	India	17	41%	88%	0.8 (0.3/1.3)	3 (1/3.5)	*Aspergillus*	Lungs	/	35%	/
Marciano et al. (59)	USA	268	69%	/	/	5.4 (3.2/11)	*Aspergillus fumigatus*	Lungs	/	17%	/
Koker et al. (60)	Turkey	89	41%	72%	/	4.2 (2.7/5.2)	*Aspergillus*	Lungs	23%	10%	/
Fattahi et al. (61)	Iran	93	13%	62%	(0.5/1.7)	(0.9/5.8)	*Aspergillus fumigatus*	Lungs	56%	10%	severe lymphadenopathy
van den Berg et al. (2)	Europe	429	67%	82%	/	(4.9/8.8)	*S. aureus*	Lungs	8%	20%	/
Jones et al. (50)	UK	94	81%	93%	/	2.7	*Aspergillus*	Lungs	/	12%	/
Wolach et al. (51)	Israel	38	29%	68%	/	/	*S. aureus*	Lungs	/	26%	recurrent pneumonia
Martire et al. (62)	Italy	60	65%	97%	0.6	2.5 (2/5.5)	*Aspergillus*	Lungs	/	13%	pneumonia
Agudelo-Florez et al. (63)	Latin America	14	/	64%	/	/	/	Lungs	0%	/	/
Carnide et al. (64)	Brazil	18	70%	89%	/	1.1	/	Lungs	/	33.00%	pneumonia
Liese et al. (65)	Germany	39	82%	95%	0.7 (0.3/1.1)	5.4 (3.8/13.6)	*S. aureus*	Lungs	/	20%	lymphadenitis
Winkelstein et al. (3)	USA	368	76%	86%	/	(3.0/7.8)	*Aspergillus*	Lungs	/	18%	/
Hasui et al. (66)	Japan	221	/	88%	/	/	/	Lungs	/	23%	/

XL, X-linked; AR, autosomal recessive; CGD, chronic granulomatous disease.

observation reported in our case series is that 13% of XL-CGD patients, but none of the AR-CGD patients had a central nervous system (CNS) abnormality. Common abnormalities under CNS include upper motor neuron dysfunction, headache, spinal cord compression, choroid plexus cyst, and unusual CNS infection, including CNS aspergillosis. The frequency of CNS aspergillosis only accounts for less than 5% in overall infections, and it has been shown that there is no significant association between the genotype and CNS aspergillosis in previous literature (53, 54). Further investigations need to be done to see whether these CNS and integumentary abnormalities are primary defects or complications of CGD or unrelated with CGD. Nevertheless, these new findings might be useful clinical handles for clinical immunologist to distinguish between XL and AR-CGD. Whenever clinicians observe some redflags, i.e., more frequent cutaneous or perianal abscesses or CNS abnormalities among CGD patients, they should suspect XL-CGD and perform targeted gene Sanger sequencing and DHR as soon as possible to confirm the diagnosis.

In addition to more frequent perianal and cutaneous abscesses seen in XL-CGD patients, a higher frequency of elevated hepatic transaminase was noted among XL-CGD patients in phenomic analysis. Previous literature has shown that abnormal liver enzymes level is common among CGD patients, occurring with at least one episode among 73% of the patients (55). However, no study has been done to correlate with the mode of inheritance and elevated hepatic transaminase. It has been hypothesised that XL-CGD patients had hepatosplenomegaly, liver abscesses, and BCGosis more often, which is a common cause of abnormal liver enzymes shown in previous literature (55). Therefore, clinicians can use the frequency of elevated hepatic transaminase to help differentiate between XL-CGD and AR-CGD.

Another notable finding in this case series is that more XL-CGD patients presented with BCGitis/BCGosis as first manifestation compared with AR-CGD patients. It has been documented that CGD patients are more prone to disseminated or local BCG infection due to defective intracellular mycobacterial killing mechanism (18, 56). BCG-related disease has been documented as a common first sign of CGD but in-depth study between genotypes of CGD has not been done. BCGitis is seen more commonly in countries where BCG vaccination is included in universal vaccination programme like mainland China, Iran, and Latin America as shown in **Table 3** (67). It has been hypothesised that patients with XL-CGD had poorer control of BCG as compared with AR-CGD and hence physicians can recognise the BCG-related disease more often as their first manifestation.

The main limitation of our study is that the clinical data provided to the APID network might be insufficient. Some of the CGD patients from our study were genetically diagnosed 20 years ago, during which the awareness and understanding of CGD was still inadequate in many countries, leading to an underreporting of CGD patients with atypical features. The authors do not have full access to the complete medical records of the CGD patients and hence some major phenotypic data of the CGD patients in our case series may be missed. Microbiological culture tests were not performed in some cases, leading to omissions in our infection profile. *Staphylococcus aureus*, for example, has been reported to be the most common pathogen causing skin abscesses in previous CGD case series but was not shown in our study. In addition, the clinical data provided to us were only up to the time when the patient was clinically diagnosed with CGD, and hence no follow-up clinical data could be computed and analysed. As a result, survival and death analysis cannot be done. As NBT or DHR assays were not always available and our cases came from many centres with different testing methodologies (68), therefore the functional phenotype of residual reactive oxygen species production could not be analysed in our case series. Since there were only 23 AR-CGD cases with sufficient clinical information, there might not be enough power resulting in false-negative results in our phenomic analysis comparing between AR and XL-CGD.

In conclusion, more severe integument infections, CNS, and hepatic enzyme abnormalities were observed in XL-CGD patients compared with AR-CGD patients. A summary of key findings regarding the differences between previous case series and this case series is presented in **Table 3**. Whenever clinicians identify such phenomic features among our children suspected to have IEI, they should suspect a diagnosis of XL-CGD and perform DHR as soon as possible. This can help speed up the diagnostic process and hence start prophylactic treatment as well as offering targeted genetic testing.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of ethical restrictions. Requests to access the datasets should be directed to lauylung@hku.hk.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Authority Hong Kong West Cluster-University of Hong Kong Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YL conceptualised the study. YL and DL designed the study. K-WC and C-YW performed genetic study. TC, HY, K-WC, and DL curated mutations. TC and DL phenotyped the patients, analysed data, and penned the manuscript. Other authors referred patients and provided clinical care and clinical data. All authors critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Pediatric Prediction Model for Low Immunoglobulin G Level Based on Serum Globulin and Illness Status

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Hypogammaglobulinemia is a condition that requires prompt diagnosis and treatment. Unfortunately, serum immunoglobulin (Ig) measurements are not widely accessible in numerous developing countries. Serum globulin is potentially the best candidate for screening of low IgG level (IgGLo) due to its high availability, low cost, and rapid turnover time. However, multiple factors may influence the probability of prediction. Our study aimed to establish a simple prediction model using serum globulin to predict the likelihood of IgGLo in children. For retrospective data of patients who were suspected of having IgGLo, both serum IgG and globulin were simultaneously collected and measured. Potential factors interfering with serum globulin and IgG levels were investigated for their impact using bivariate binary logistic regression. A multivariate binary logistic regression was used to generate a formula and score to predict IgGLo. We obtained 953 samples from 143 pediatric patients. A strong positive correlation between serum globulin and IgG levels was observed ($r=0.83$, $p < 0.001$). A screening test model using serum globulin and illness status was constructed to predict IgGLo. The formula for predicting IgGLo was generated as follows; Predicted score = (2 x globulin (g/dl)) – illness condition score (well=0, sick=1). When the score was <4, the patient has the probability of having IgGLo with a sensitivity of 0.78 (0.71, 0.84), a specificity of 0.71 (0.68, 0.74), PPV of 0.34 (0.29, 0.40) and NPV of 0.94 (0.92, 0.96). This formula will be useful as rapid and inexpensive screening tool for early IgGLo detection, particularly in countries/locations where serum IgG measurement is inaccessible.

Keywords: globulin, hypogammaglobulinemia, prediction model, screening test, immunoglobulin G

Abbreviations: Ig, immunoglobulin; IgGLo, low IgG level; IEI, inborn errors of immunity; ESID, European Society for Immunodeficiencies; IVIG, intravenous immunoglobulin; ROC, receiver operating characteristic; NPV, negative predictive values; PPV, positive predictive values; CID, combined immunodeficiency; AUC, area under the curve.

INTRODUCTION

Hypogammaglobulinemia refers to a reduction in all types of immunoglobulins (Ig). This condition is strongly associated with recurrent serious infections with encapsulated pyogenic bacteria, including *Streptococcus pneumoniae* and *Haemophilus influenzae type b* (1). IgG is the most abundant idiotype in the circulation. Low IgG level (IgGLo) is defined as a decrease in IgG concentrations at least 2 standard deviations below the mean for the age group (2). Diseases underlying hypogammaglobulinemia and IgGLo may vary from inborn errors of immunity (IEI) (3) to acquired causes (malignancies, severe infections, malnutrition, excessive protein loss from the gastrointestinal tract, skin, and kidneys, or as a side effect of medications) (4, 5). Prompt diagnosis of IgGLo and subsequent treatment with IgG replacement therapy is crucial to prevent long-term morbidity and death (6, 7).

In case of suspected hypogammaglobulinemia, international consensus guidelines recommend prompt measurement of serum IgG (8–10). However, in numerous developing countries, such tests are only available in referral centers or tertiary care hospitals. Therefore, the availability of a simple and inexpensive tool that is widely accessible and applicable would be beneficial, especially for shortening the diagnostic delay of IgGLo. Serum globulin levels, routinely measured in liver function testing, typically yields information on three globulin fractions, namely alpha, beta, and gamma globulins. Serum Igs, in particular IgG, constitutes a significant part of the gamma globulin fraction. Therefore, in developing countries, measurement of serum globulin levels is an attractive candidate to screen for IgGLo due to its high availability, low cost, and rapid turnover time.

A few studies have reported a positive correlation between serum globulin and IgG levels, and demonstrated the feasibility of using serum globulin level as a screening test for hypogammaglobulinemia (8, 9, 11). However, these studies did not consider potential factors that might influence the accuracy of prediction. The main influential factor is serious infection accompanied by an increase in complement proteins, which in turn raises serum globulin levels (9). The simple formula that is suitable for clinical settings has never been established. Also, Data on pediatric populations are limited (12). Moreover, a simple formula that can be applied in clinical settings has never been established. Our study aimed to establish a simple and rapid formula based on inexpensive serum globulin measurement that can be implemented in a clinical setting to predict the probability of IgGLo in patients <18 years of age.

MATERIAL AND METHODS

Patients

Medical records from the period of 2011-2021 of patients under 18 years of age (Department of Pediatrics, King Chulalongkorn Memorial Hospital, Bangkok, Thailand) with suspected hypogammaglobulinemia or IgGLo were reviewed. Patients were enrolled in this study when serum IgG and globulin levels measured from the same time point were available. Age, sex, causes of hypogammaglobulinemia, intravenous immunoglobulin (IVIG) replacement, and illness conditions from the time of blood sample collection were retrieved from the medical files. This study was approved by the Ethics Committee of King Chulalongkorn Memorial Hospital, Bangkok, Thailand (IRB No. 504/59).

Operating Definition

Low Serum IgG Levels (IgGLo)

In general, IgGLo is defined as a decrease in IgG concentrations at least 2 standard deviations (SDs) compared to mean age-specific IgG level. However, the risk of recurrent and severe infections generally occurs particularly when serum IgG levels are <500 mg/dl (13). Therefore, in our study, IgGLo was defined as a serum IgG level <500 mg/dl, regardless of age.

The Causes of IgGLo

The causes of IgGLo were divided into main two categories: primary and secondary causes. Primary causes of IgGLo related to IEI, particularly B cell differentiation defects. Secondary or acquired causes were assigned to patients when diseases or other extrinsic factors related to IgGLo were identified. These categories are: (1) loss of IgG, including burns, congenital lymphangiectasia, and nephrotic syndrome; (2) drugs that inhibit IgG production or lead to increased IgG metabolization, such as corticosteroids, immune suppressants, and anticonvulsants; (3) malignancies or collagen vascular diseases; (4) viral infections, including Epstein-Barr virus, rubella, HIV, and cytomegalovirus; and (5) other causes related to IgGLo, such as severe malnutrition, severe infections, and prematurity (14).

Illness Conditions

Two illness conditions were classified in our study. "Sick" referred to the condition in which the patient had a fever, was admitted to the hospital, or received antibiotics for a treatment at the time blood samples were drawn for IgG and globulin measurement. "Well" referred to the condition in which the individual was in good health when blood samples were drawn.

Intravenous Immunoglobulin (IVIG) Replacement Therapy

The half-life of plasma IgG from IVIG is approximately 26–41 days (15). It usually takes 4-5 half-lives to clear the majority of IVIG from the body. IVIG replacement affects vaccination responses for six months after the last dose (16). So in our study, a sample was defined as receiving IVIG replacement when the time of blood sample collection was within six months after the last dose of IVIG.

Blood Sample Measurement

The globulin fraction (g/dl) was obtained as part of the liver function test, which was determined from the difference between serum total protein and albumin levels. Total protein and albumin levels were measured by the architect biuret method and colorimetric bromocresol green method, respectively. Serum IgG levels (mg/dl) were determined using nephelometry. Both serum IgG and globulin were measured at the same collection time.

Statistical Analysis

The characteristics of the included patients were described using means and SDs for continuous variables, counts, and percentages for categorical characteristics. Pearson's correlation was used to measure the strength and direction of the association between serum globulin and IgG levels. Then we formulated the model for predicting IgGLo from serum globulin. Firstly, potential factors that might interfere with serum globulin and IgG levels were selected and investigated using bivariate binary logistic regression. Potential diagnostic factors with a p-value less than 0.2 in the bivariate analysis were then included in a multivariable binary logistic regression and were removed if they were not statistically significant. After identifying the significance of the diagnostic factors and generated models, their utility in diagnosing IgGLo was investigated using receiver operating characteristic (ROC) curves. We fitted the diagnostic model with the variables and removed individual variables to examine whether it reduced model accuracy. Once we decided on the final model, we simplified the model for practical use in clinical situations by rounding the coefficients and comparing the ROC curves of the original and simplified models. We generated the sensitivity, specificity, negative predictive values (NPV) and positive predictive values (PPV), and negative and positive likelihood ratios for the simplified model. Finally, we simplified the cut-off point and investigated whether the diagnostic accuracy of the model was changed. All analyses were conducted using the R statistical package (R core team, 2017); (17, 18), and ROC analysis using the R library pROC (19). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Demographic Data

Demographic data of the patients and samples collected are provided in **Tables 1** and **S1**. In this study, 953 samples from 143 patients were included. Sixty-nine percent of the participants were male. The mean age ± SD of patients having IEI was 7.8 ± 5.1 years old, while the mean age ± SD of patients with acquired IgGLo was 4.76 ± 5.0 years old. Seventy-six percent of the samples were collected from patients with IEIs, half of whom had predominantly antibody deficiencies. The leading cause of acquired IgGLo was sepsis (40.3%). Eighty percent of IEI samples were collected while the patients were in good health, while around sixty percent of samples with acquired IgGLo were collected when the patients were getting sick. Ninety percent of IEI samples and 75 percent of samples with acquired IgGLo were drawn when IVIG was given.

Correlation Between Serum Globulin and IgG Levels

The association between serum globulin and IgG levels is shown in **Figure 1**. Our results demonstrated a strong positive

TABLE 1 | Demographic Characteristics of Patients and Samples.

Characteristic	Inborn Errors of Immunity (IEI)				Secondary immunodeficiency				Total
	Ab def	Combined	Others	Total subgroup	Sepsis/severe infections	Recurrent pneumonia	Hematologic disorders	Total subgroup	
Patients (Total numbers = 143)									
No. of patients; n (%)	16 (11.2)	13 (9.1)	5 (3.5)	34 (23.7)	43 (30.1)	27 (18.9)	39 (27.3)	109 (76.2)	**143 (100)**
Age of the patients; mean years (SD)	6.4 (5.2)	2.9 (4.2)	4.0 (4.1)	4.7 (4.8)	2.8 (4.2)	6.2 (4.1)	4.4 (4.6)	4.2 (4.5)	**4.3 (4.5)**
Male sex; n (%)	11 (44.0)	10 (40.0)	4 (16.0)	25 (73.5)	31 (41.9)	16 (21.6)	27 (36.5)	74 (67.9)	**99 (69.2)**
Patients receiving IVIG; n (%)	15 (93.8)	8 (61.5)	1 (20.0)	24 (79.4)	11 (25.6)	3 (11.1)	1 (2.6)	15 (13.8)	**39 (27.3)**
Samples (Total numbers = 953)									
No. of samples; n (%)	411 (43.1)	251 (26.3)	63 (6.6)	725 (76.1)	92 (9.7)	73 (5.2)	63 (6.6)	228 (23.9)	**953 (100)**
Sick condition; n (%)	42 (10.2)	84 (33.5)	8 (12.7)	134 (18.5)	75 (81.5)	42 (57.5)	23 (36.5)	140 (61.4)	**274 (28.8)**
Sample obtained during IVIG administration; n (%)	393 (96.6)	231 (94.7)	53 (100)	677 (93.4)	33 (80.5)	9 (60.0)	3 (23.1)	45 (19.7)	**722 (75.8)**
Serum globulin levels before IVIG administration (g/dl); mean (SD)	1.6 (0.5)	2.4 (0.9)	N/A	2.1 (0.8)	1.5 (0.5)	2.3 (0.8)	2.8 (-)	1.8 (0.7)	**1.9 (0.8)**
Serum globulin levels after IVIG administration (g/dl); mean (SD)	2.2 (0.4)	2.8 (0.9)	2.3 (0.3)	2.4 (0.7)	2.1 (0.7)	2.5 (0.7)	4.2 (0.5)	2.3 (0.9)	**2.4 (0.7)**
Serum IgG levels before IVIG administration (mg/dl); mean (SD)	122.9 (89.5)	453.1 (432.2)	N/A	333.0 (377.1)	324.7 (242.3)	1054.7 (673.0)	1330 (-)	591.0 (528.7)	**467.6 (470.9)**
Serum IgG levels after IVIG administration (g/dl); mean (SD)	686.2 (170.2)	1091.6 (565.0)	693.6 (109.2)	825.1 (404.0)	746.3 (524.2)	823.6 (507.9)	1943.3 (406.7)	841.6 (586.4)	**826.2 (417.1)**

IgG, immunoglobulin G; IVIG, intravenous immunoglobulin; N/A, not available; Ab def, predominantly antibody deficiencies; Combined, immunodeficiencies affecting cellular and humoral immunity; Others, other IEIs including congenital defects of phagocyte and combined immunodeficiencies with associated or syndromic features.
The bold numbers mean for the numbers of the total groups.

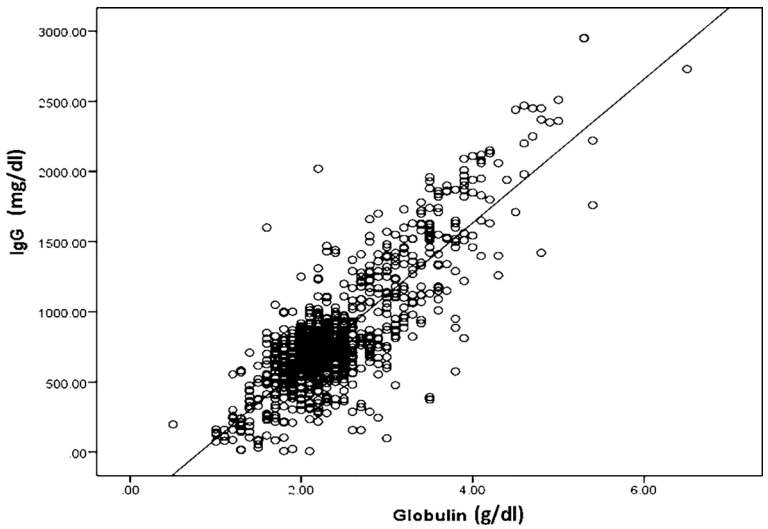

FIGURE 1 | A scatter plot showing a strong positive correlation between serum globulin levels and serum immunoglobulin G levels in all 953 serum samples; $r^2 = 0.83$, $p < 0.001$.

correlation between serum globulin and IgG levels (r=0.83, p <0.001). Subgroup analysis of 725 samples from patients with IEI and 228 samples from patients with acquired IgGLo also showed a strong correlation between serum globulin and IgG levels (r=0.84, p < 0.001 and r=0.82, p < 0.001, respectively). However, among patients with IEI, patients who had immunodeficiencies affecting cellular and humoral immunity, and other IEIs including congenital defects of phagocyte and combined immunodeficiency (CID) with associated or syndromic features, a stronger correlation was observed (r=0.89, p < 0.001 and r= 0.86, p < 0.001, respectively), while a weaker correlation between serum globulin and IgG levels (r=0.48, p < 0.001) was observed in patients with predominantly antibody deficiencies.

Prediction Models for IgGLo

Considering that multiple factors can influence serum globulin and IgG levels, we gathered information regarding potential factors including patient age, sex, IVIG replacement, the causes of IgGLo and the illness conditions when serum IgG and globulin levels were determined. We found that patient age, illness conditions, and IVIG replacement were the significant factors that affected serum globulin and IgG levels (p = 0.01, p < 0.001, and p < 0.001 respectively). Using ROC analysis, the area under the curve (AUC) of the model to predict IgGLo taking all significant factors into accounts was 0.8705(0.838, 0.903). When the IVIG replacement was removed, the AUC of the model was 0.8497 (0.815, 0.884). Although the AUC of both models were significantly different (p= 0.0046), the magnitude of difference is quite small (0.02 or 2%). Therefore, IVIG replacement therapy was not included in the final model.

Different models from serum globulin, patient age and illness conditions were created and tested for accurate prediction of IgGLo. The models and performance characteristics, including sensitivity, specificity, NPV, and PPV, are provided in **Table 2**. ROC curves illustrate the diagnostic ability of models (**Figure 2**).

The first prediction model generated was "Predictive score = (- 0.05 x age) + [- 3 x globulin (g/dl) + (1.3 x illness condition score); while well=0, sick=1]" which we further simplified to "Predictive score = age (years) + [60 x globulin (g/dl)] – (25 x illness condition score)". When the predictive score was ≤ 127.6, the model could predict IgGLo with a sensitivity of 0.77 (0.69, 0.83), specificity of 0.79 (0.76, 0.81), PPV of 0.41 (0.35, 0.47) and NPV of 0.95 (0.93, 0.96). We observed that the removal of "age of patient" from the model did not affect the model performance, however, it provided more simplicity. Therefore, the new model

TABLE 2 | Performance characteristics of the different models for diagnosing low IgG levels.

Model for calculated predictive score (x)	Cutoff score	Sensitivity (95%CI)	Specificity (95%CI)	PPV	NPV
X = age + (60 x globulin) – (25 x I)	127.6	0.77 (0.69, 0.83)	0.79 (0.76, 0.81)	0.41	0.95
X = (-2.85 x globulin) + (1.62 x I)	5.6	0.77 (0.69,0.83)	0.79 (0.76,0.82)	0.42	0.95
X = (2 x globulin) – I	3.9	0.75 (0.68, 0.82)	0.80 (0.77, 0.83)	0.42	0.94
X = (2 x globulin) – I	4.0	0.78 (0.71, 0.84)	0.71 (0.68, 0.74)	0.34	0.94

X, predictive score; I, illness condition score (well= 0, sick= 1); PPV, positive predictive value; NPV, negative predictive value. Age was described in years, the unit of globulin level was g/dl; IgG, immunoglobulin G.

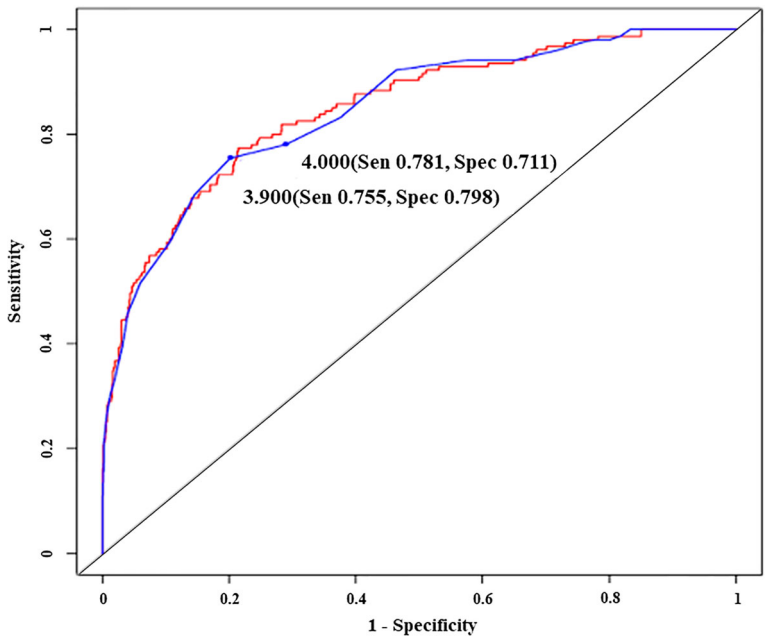

FIGURE 2 | Receiver operating characteristic curves illustrating the diagnostic ability of an original model (red line) and a simplified model with two different cut-off predictive scores (blue line), Sen, sensitivity; Spec, specificity. - Original model; Predictive score = -2.85 x globulin (g/dl) + (1.62 x illness condition score) - Simplified model; Predictive score = 2 x globulin (g/dl) - illness condition score - Illness condition score (well= 0, sick= 1).

generated without considering patient age was "Predictive score = -2.85 x globulin (g/dl) + (1.62 x illness condition score)". With the predictive score of ≤ 5.6, the diagnostic performance of the later model was as follows; sensitivity of 0.77 (0.69, 0.83), specificity of 0.79 (0.76, 0.82), PPV of 0.42 (0.36, 0.48) and NPV of 0.95 (0.93, 0.96).

We again rounded up the coefficients of the model to "Predictive score = 2 x globulin (g/dl) - illness condition score". With a predictive score of ≤ 3.9, the performance of the diagnostic model remained good, with a sensitivity of 0.75 (0.68, 0.82), specificity of 0.80 (0.77, 0.83), PPV of 0.42 (0.36, 0.48) and NPV of 0.94 (0.92, 0.96). Finally, we rounded up the predictive score to the "4" and tested whether the diagnostic value of the model was compromised. Our result showed that with the predictive score ≤ 4, the probability to have IgGLo was not compromised, with a sensitivity of 0.78 (0.71, 0.84), specificity of 0.71 (0.68, 0.74), PPV of 0.34 (0.29, 0.40) and NPV of 0.94 (0.92, 0.96).

DISCUSSION

Delay in the recognition of IgGLo leads to diagnostic and therapy delay, resulting in devastating consequences. Limited access to IgG measurement is a major reason for delayed diagnosis of this condition in developing countries where measurement of serum IgG levels can be performed only in referral centers. The ultimate goal of this study was to establish a realistic model to predict IgGLo in a clinical setting. We carefully investigated factors potentially influencing IgGLo and generated different models to obtain the most appropriate model and cut-off score to predict IgGLo.

Serum IgG are the major constituents of the serum gamma globulin fraction. Therefore, serum globulin measurement could be an attractive candidate for screening IgGLo. Using serum globulin as a first screening tool is practical because of its high availability, low cost, and rapid turnover time. Previous studies have shown a strong correlation between serum globulin and IgG (8, 9). These studies also proposed serum globulin cut-off levels for predicting IgGLo (8, 9). However, data on children are scarce (12). Importantly, we believe that factors other than serum globulin should also be taken into account for proper prediction of IgGLo. Our results demonstrate that illness condition is an important factor that influences the diagnostic model and performance in children. This is in line with the fact that acute phase proteins are increasing during acute infection and therefore have an impact on the serum globulin level measured under such condition (20). We found no relationship between the causes of IgGLo and the correlation between serum globulin and IgG, making the model more widely applicable.

Even though serum IgG levels in children under one year of age might be influenced by maternal IgG, the age of the patient at the moment of sample collection did not significantly affect our prediction model. In our study 12.3% of samples (117 out of 953 samples) were collected from patients under one year of age (33% of total numbers of patients included). Our analysis revealed that the predictive accuracy of the model was not compromised when the samples obtained at age < 1 year were included. We consider

this important since certain patients with IEI with low IgG levels present at very early age (such as severe combined immunodeficiency or X-linked agammaglobulinemia). Therefore, the result of our study is beneficial in early detection of these populations, especially in places where measurement of serum IgG levels is not available.

The reason why the correlation between serum globulin and IgG levels was weaker in patients with predominant antibody deficiencies compared to the other IEI subgroups remains unclear. Additionally, although not proven in our study, some conditions can interfere with the components of serum globulin [e.g. hyperlipoproteinemia (increase alpha-1 globulin), metastatic malignancy (increase alpha-1 and alpha-2 globulin), alpha-1 antitrypsin deficiency (decrease alpha-1 globulin), hemoglobin-haptoglobin complexes secondary to hemolysis (increase alpha-2 globulin), and iron-deficiency anemia with high transferrin (increase beta globulin)] (21). Thus, they may compromise the predictive accuracy for low IgG levels.

In conclusion, early diagnosis of IgGLo is crucial. Serum globulin measurement is a rapid, simple, inexpensive, and widely available tool for predicting IgGLo. We constructed the following screening model/formula for predicting IgGLo in children (age <18 yrs): "Predicted score = (2xglobulin (g/dl)) – illness condition score (well= 0, sick= 1)", with a score of ≤4 being predictive for IgGLo. We propose that application of this simple and cheap model in developing countries where measurement of serum IgG is generally unavailable would be a valuable tool to reduce diagnostic delay and optimize the use of healthcare resources for the diagnosis of IgGLo in children (age <18 years).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to pantipa.c@chula.md.

ETHICS STATEMENT

This study was approved by the Ethics Committee of King Chulalongkorn Memorial Hospital, Bangkok, Thailand, IRB No. 504/59. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NS wrote the first draft of manuscript, collected and analyze data. PT collected data, analyzed the data and wrote the manuscript. CH and YC performed the statistical analysis and wrote the manuscript. JW performed serum globulin and IgG testing and wrote manuscript. PH and WD wrote the manuscript. PC conceptualized of the study and wrote manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.825867/full#supplementary-material

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frontiers in Immunology

CASE REPORT
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Case Report: Rotavirus Vaccination and Severe Combined Immunodeficiency in Japan

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Severe combined immunodeficiency (SCID) is an inborn error of immunity that occurs in approximately 1 in 50,000 births, mainly due to impaired lymphocyte differentiation. Without curative treatment, such as hematopoietic cell transplantation (HCT) or gene therapy, severe infection in the first year of life could make this condition fatal. The results of HCT are poor when patients have active infections, thus requiring early diagnosis before onset of infection. In five cases of SCID diagnosed in Japan, the oral rotavirus vaccine had been administered before diagnosis. In this study, we demonstrated that the rotavirus from their stools was a vaccine-derived strain. In some cases, severe gastroenteritis triggered the diagnosis of SCID. However, newborn screening for SCID is available before the first rotavirus vaccination using assays for the detection of T-cell receptor excision circles (TRECs). Therefore, to improve the prognosis of patients with SCID in Japan, we should establish a screening system of TRECs for newborns throughout Japan.

Keywords: severe combined immunodeficiency, hematopoietic cell transplantation, rotavirus, vaccination, T-cell receptor excision circles (TREC)

INTRODUCTION

Severe combined immunodeficiency (SCID) is a critical inborn error of immunity (IEI) that causes cellular and humoral immunity failure due to impaired lymphocyte differentiation, resulting in severe infections from infancy. Therefore, hematopoietic cell transplantation (HCT) or gene therapy is required as a curative therapy.

Rotavirus infection is a gastrointestinal infection that occurs in infants and children. Although mild cases can resolve spontaneously, some cases can cause fatal dehydration and encephalitis or

encephalopathy, leading to the hospitalization of many patients. Although live vaccination is contraindicated (1), patients with SCID often remain asymptomatic until early infancy and are rarely diagnosed before 2 months of age when oral rotavirus vaccination is initiated. Furthermore, Rotarix®, a live, monovalent, attenuated, human rotavirus vaccine (RV1), and RotaTeq®, a live, pentavalent, human–bovine reassortant rotavirus vaccine (RV5), were initiated as arbitrary vaccination in 2011 and 2012, respectively, in Japan. Regular administration of these vaccines was initiated in October 2020. This initiative was taken after realizing that the number of cases vaccinated before the diagnosis of SCID will increase.

Several severe cases of rotavirus gastroenteritis caused by vaccine strains in SCID have been reported overseas (2, 3) as well as in Japan (4, 5). This report summarizes the cases of five patients with SCID who were vaccinated before being diagnosed with SCID in Japan.

MATERIALS AND METHODS

RNA Extraction from Patient Stool and Serum

Ten percent suspensions (1 ml) of each stool sample were prepared in physiological saline solution or swab samples were rinsed in 500 µl of physiological saline solution. Each suspension was then centrifuged for 20 min at 4,000 × *g* and 140 µl of the supernatant was used for RNA extraction. Finally, viral double-stranded RNAs (dsRNAs) were extracted from the stool suspension and 140-µl sera using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany).

Sanger Sequencing

First, RNA was extracted from patients' stool samples and RotaTeq and complementary DNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Foster City, CA, USA). DNA was amplified by polymerase chain reaction (PCR) using primers for human rotavirus A gene 10, which encodes the rotavirus *Non-structural protein 4* (*NSP4*) gene, and sequenced using the Sanger method (forward primer: 5'-GGGCTTTTAAAAGTTCTGTTCCGAG-3', reverse primer: 5'-GGTCACACTAAGACCATTCC-3'). Finally, we compared nucleotide and amino acid sequences of rotavirus strains derived from patients' stool samples, the RotaTeq WC3 strain, and the wild-type human/Wa strain.

RNA Extraction and RV1- and RV5-Specific Real-Time Reverse Transcription-Polymerase Chain Reaction

The stool samples were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for the presence of RV5, RV1, and wild-type strains (6), and details about the real-time RT-PCR analysis were described elsewhere (7). Real-time RT-PCR was performed on a Fast Optical 48-Well Reaction Plate using a TaqMan RNA-to-Ct 1-Step kit (Thermo Fisher Scientific, Waltham, MA). In addition, single-well denaturation, reverse transcription, and amplification were performed on a StepOne Real-Time PCR system in standard mode (Thermo Fisher Scientific). Thermocycling conditions included a 15-min hold at 48°C, a 10-min cycle at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Each rotavirus vaccine strain-specific real-time RT-PCR amplified the designated vaccine strain only, and no cross-reaction was observed with any of the wild-type strains. Meanwhile, real-time RT-PCR to detect *Non-structural protein 3* (*NSP3*) gene in wild-type strains could also detect the two vaccine strains. Serially diluted purified rotavirus virions were used to determine the lower detection limits of RV5 (10 copies/reaction), RV1 (50 copies/reaction), and wild type (50 copies/reaction) using real-time RT-PCR. While the RNA extracted from RV5 and RV1 was used as a positive control for vaccine virus strains, the KU strain (G1P [8]) was used as a positive control for the wild-type virus.

RESULTS

Case Reports

Cases were collected throughout Japan from 2015 to 2020 (**Figure 1** and **Table 1**). The first case (P1) was that of a patient with X-linked SCID, interleukin (IL)-2 receptor gamma (CD132) deficiency, who received the first dose of RV5 at 8 weeks of age (5, 8). He developed respiratory syncytial virus infection and interstitial pneumonia at 5 months of age and was admitted to the hospital. Upon recovery after a 3-week treatment, he had splenomegaly and hypergammaglobulinemia (immunoglobulin G (IgG) > 3,000 mg/dl). Further examination revealed natural killer (NK)-cell deficiency and skewing to memory $CD4^+$ T cells, and IEI was suspected. Genetic analysis revealed the causative agent to be the *IL2RG* variant (c.676C>T, p.R226C). However, while preparing for HCT, the patient experienced loose stools, and a rapid test showed that he was rotavirus-positive. Cord blood transplantation (CBT) was performed at 11 months of age at Tokyo Medical and Dental University. The patient presented with loose stools from day 0 to day 11 after CBT. Rotavirus antigen test turned negative on day 28.

The second case (P2), also with X-SCID, was administered the first dose of RV1 at 9 weeks of age. He developed severe *Pneumocystis* pneumonia (PCP) at 4 months, requiring mechanical ventilation. Flow cytometry revealed NK-cell deficiency with maternal T cells and lack of CD132 on lymphocytes, and the patient was diagnosed with X-SCID with the *IL2RG* variant (c.202 G>T, p.E68*). Although gastrointestinal symptoms were not the main symptoms, the patient tested positive for the rotavirus antigen in the screening test on admission. The patient underwent CBT at 6 months of age (27 weeks 1 day) at the National Center for Child Health and Development. Rotavirus antigen test turned negative 32 days after CBT.

The third case (P3) was that of a patient with X-SCID who was vaccinated with RV5 at 9 weeks of age (4). Three vaccine doses were administered; however, the patient was admitted to

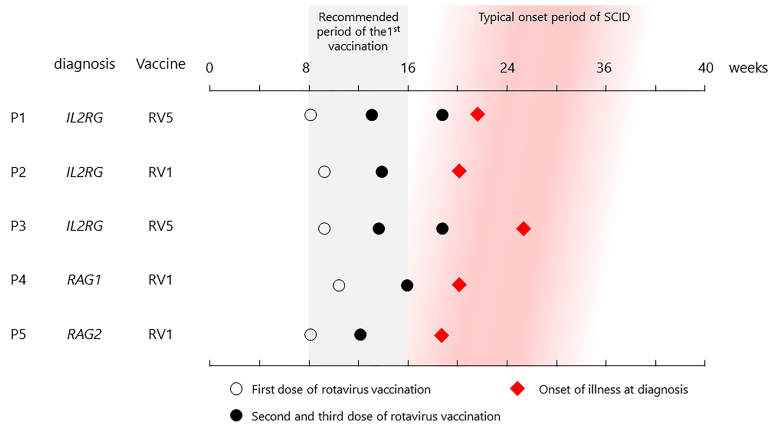

FIGURE 1 | List of vaccination and onset dates in patients. All patients received their first vaccine dose within the recommended period, and each showed symptoms at the typical onset time.

the hospital because of diarrhea and weight loss (max −15%) at 5 months. He also had agammaglobulinemia, a lack of T cells and CD132, and was diagnosed with X-SCID (*IL2RG*: c.74_75insAC, p.T26Rfs). The patient underwent CBT at 9 months of age (42 weeks 6 days) at Hokkaido University; however, he remained positive for rotavirus antigen until then. Rotavirus antigen turned negative 27 days after CBT, and steady body weight gain was achieved since then.

The fourth case (P4) was that of a boy with recombination activating gene 1 (RAG1) deficiency, an autosomal recessive SCID, and was vaccinated with RV1 at 10 weeks of age. He was initially admitted to the Kyushu University Hospital at 4 months of age with rhinovirus infection and interstitial pneumonia and was then diagnosed with RAG1 deficiency (*RAG1*: c.2209C>T, p.R737C and c.2923C>T, p.R975W). In addition, he had loose stools and was positive for the rotavirus antigen. He underwent bone marrow transplantation (BMT) from HLA-matched sibling at 6 months of age (26 weeks 0 days). Rotavirus antigen test turned negative 15 days after BMT.

The fifth case (P5) was that of a girl with autosomal recessive SCID, RAG2 deficiency (*RAG2*: c.143T>A, p.L48Q and c.419A>G, p.H140R) who developed PCP at 4 months of age after receiving RV1 (9). During the course of treatment at Nagoya University Hospital for disease management, vomiting and increased gastric remnants were observed, and she was diagnosed as having rotavirus antigen-positive gastroenteritis. Thus, the first CBT was performed at 8 months of age without conditioning treatment because of severe PCP. Although she had mix chimerism, rotavirus antigen test turned negative 38 days after the first CBT. The second CBT with conditioning regimen was performed at 17 months, and she achieved full chimerism and revolved PCP completely.

In all cases, each patient completed the default doses of RV1 or RV5. One case (P2) did not demonstrate GI symptom, and rotavirus antigen was incidentally identified by screening on admission. Two cases (P1 and P4) had minor symptoms with loose stools, and one (P5) had mild symptoms with vomiting. They had no weight loss due to GI symptoms. In contrast, one case (P3) presented with severe diarrhea and weight loss. Total parenteral nutrition was not required in any of them.

Detection of Vaccine-Derived Rotavirus

Further examination of the rapid test-positive feces revealed that the rotavirus strain from P1 stool was the vaccine-derived strain

TABLE 1 | Clinical features of 5 cases.

	P1	P2	P3	P4	P5
Gene	*IL2RG*	*IL2RG*	*IL2RG*	*RAG1*	*RAG2*
Variants	c.676C>T p.R226C	c.202 G>T p.E68*	c.74_75insAC p.T26Rfs	c.2209C>T p.R737C c.2923C>T p.R975W	c.143T>A p.L48Q c.419A>G p.H140R
Vaccine	RV5	RV1	RV5	RV1	RV1
Total dose	3	2	3	2	2
Severity of GI symptoms	Mild	None	Severe	Mild	Mild
Post HCT days when antigen test turned negative	Day 14	Day 32	Day 27	Day 15	Day 38
Weight loss due to GI symptoms	None	None	Severe	None	None
TPN	No	No	No	No	No

RV1, Rotarix®; RV5, RotaTeq®; GI, gastrointestinal; HCT, hematopoietic cell transplantation; TPN, total parenteral nutrition.

by Sanger sequence, and those from the others were vaccine strains derived by RT-PCR. Sequential data of viral loads in stools were demonstrated in P2, P3, and P4, and they decreased after HCT (**Figure 2**). In addition, the vaccine strain was detected in the serum of P2 and P4, suggesting that the disease developed into a systemic infection.

DISCUSSION

Patients with SCID are asymptomatic until 3–4 months of age when maternal IgG levels decrease, making it challenging to diagnose SCID based on physical examination and history alone without knowing family history (1). The recommended age for the first dose of rotavirus vaccine is 8–15 weeks, considering when the infection becomes severe; late administration should be avoided (10). The five SCID cases from Japan suggest that rotaviruses are continuously excreted from the intestinal tract of infected patients, even if they do not show severe gastroenteritis symptoms. Although transmission of the vaccine strain rotavirus is not considered a major problem in healthy infants (11), nosocomial infection thought to be derived from the excreted vaccine strain of rotavirus was observed in P5 (9). Therefore, live rotavirus vaccines should not be administered before diagnosing patients with SCID, even if the disease is not severe. With an annual incidence of approximately 1 in 50,000, SCID is not a rare disease compared with other diseases subjected to mass screening of newborns and given the existence of curative treatments, such as HCT and gene therapy, thereby making it appropriate for screening (12) (unfortunately, gene therapy is not available in Japan). Early diagnosis is necessary to exclude patients with SCID from live rotavirus vaccination targets. T-cell receptor excision circles (TRECs) quantitative PCR assay using neonatal dried blood spots is a useful diagnostic method (13).

Furthermore, a recent review of newborn screening with TRECs showed that the sensitivity of the test for SCID is 100%, demonstrating its usefulness (14). In 2018, TRECs screening for newborns was implemented in all states of the United States, spreading globally, including Europe. However, regional disparities in medical care is a problem because only a few regions in Japan have implemented this system. Assuming that 850,000 babies are born annually in Japan, approximately 15–20 per year will be diagnosed with SCID. Therefore, it is desirable to establish a screening system of TRECs for all newborns in Japan at an early stage.

CONCLUDING REMARK

We described five SCID cases associated with rotavirus-derived infection in Japan with some showing severe gastroenteritis. Therefore, we should introduce TRECs screening for newborns to improve prognosis of SCID in Japan.

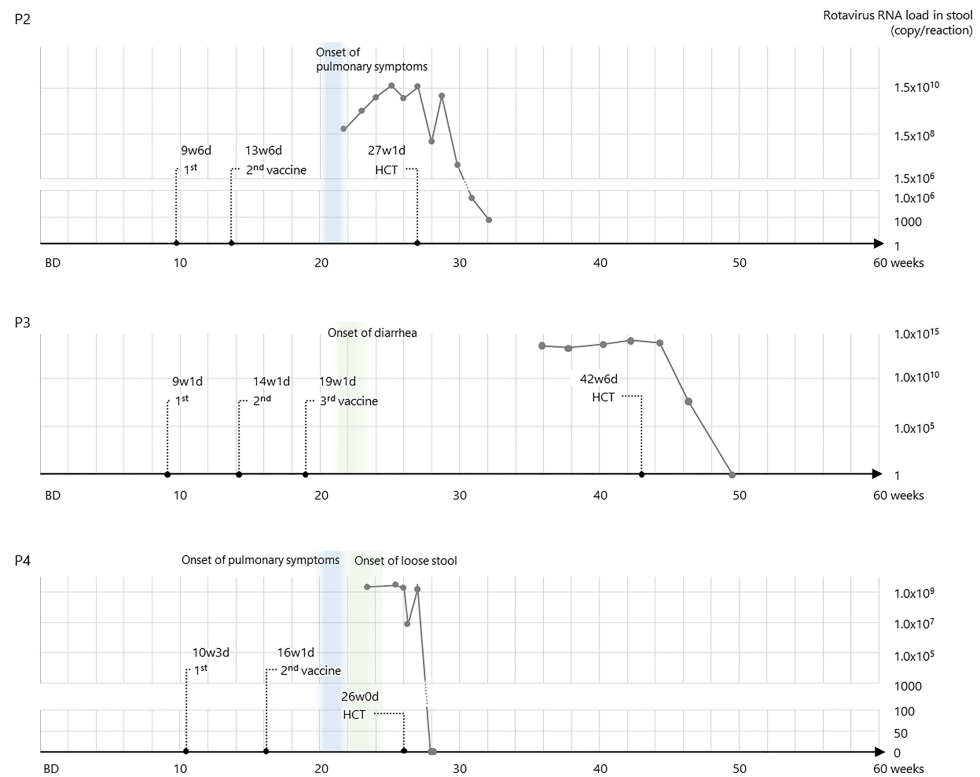

FIGURE 2 | RV5 genotype G1 RNA loads in the stool samples of patients 2, 3, and 4.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics boards of the Tokyo Medical and Dental University and Fujita Health University School of Medicine. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

KT and YK wrote the manuscript. YK, HirM, and NM performed genetic analysis. TT, KeI, AI, MY, TaY, HidM, NT, TosM, MK, KE, and MI provided clinical information. SO, KoI, and TomM provided critical discussion. TeY revised the manuscript. HK conceptualized the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Janus Kinase Inhibitors in the Treatment of Type I Interferonopathies: A Case Series From a Single Center in China

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Objective: This study aimed to assess the efficacy and safety of 2 Janus kinase (JAK) inhibitors (jakinibs) tofacitinib and ruxolitinib in the treatment of type I interferonopathies patients including STING-associated vasculopathy with onset in infancy (SAVI), Aicardi-Goutières syndrome (AGS), and spondyloenchondrodysplasia with immune dysregulation (SPENCD).

Methods: A total of 6 patients were considered in this study: 2 patients with SAVI, 1 patient with AGS1, 1 patient with AGS7, and 2 patients with SPENCD. Clinical manifestations, laboratory investigations, radiology examinations, treatment, and outcomes were collected between November 2017 and November 2021 in Peking Union Medical College Hospital. The disease score for patients with SAVI and AGS scale for patients with AGS were documented. The expression of 6 interferon-stimulated genes (ISGs) was assessed by real-time PCR.

Results: Three patients (1 patient with SAVI, 2 patients with AGS) were treated with ruxolitinib and 3 patients (1 patient with SAVI, 2 patients with SPENCD) were treated with tofacitinib. The mean duration of the treatment was 2.5 years (1.25–4 years). Upon treatment, cutaneous lesions and febrile attacks subsided in all affected patients. Two patients discontinued the corticoid treatment. Two patients with SAVI showed an improvement in the disease scores ($p < 0.05$). The erythrocyte sedimentation rate normalized in 2 patients with AGS. The interferon score (IS) was remarkably decreased in 2 patients with SPENCD ($p < 0.01$). Catch-ups with growth and weight gain were observed in 3 and 2 patients, respectively. Lung lesions improved in 1 patient with SAVI and remained stable in 3 patients. Lymphopenia was found in 3 patients during the treatment without severe infections.

Conclusion: The JAK inhibitors baricitinib and tofacitinib are promising therapeutic agents for patients with SAVI, AGS, and SPENCD, especially for the improvement of cutaneous lesions and febrile attacks. However, further cohort studies are needed to assess the efficacy and safety.

Keywords: type I interferonopathies, autoinflammatory disorders, Janus kinase inhibitors, ruxolitinib, tofacitinib

INTRODUCTION

Type I interferon (IFN)-mediated monogenic autoinflammatory (IFNopathies) disorders are recently identified as a subgroup of inborn errors of immunity, which include a genetically and phenotypically heterogeneous group of autoinflammatory and autoimmune disorders with high morbidity and mortality. They predominantly affect young child such as Aicardi–Goutières syndrome (AGS), STING-associated vasculopathy with onset in infancy (SAVI), and spondyloenchondrodysplasia with immune dysregulation (SPENCD) (1, 2). According to the last classification from the International Union of Immunological Societies Expert Committee, 15 different gene mutations correspond to 15 distinct disorders and many IFN-related diseases have yet to be discovered. IFNopathies are different from the inflammatory diseases mediated by interleukin (IL)-1 and tumor necrosis factor (TNF)-α, since they are characterized by the overproduction of type I IFN in the blood and cerebral spinal fluid, leading to the excessive activation of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway (3). Clinical manifestations are also distinct from those of the canonical autoinflammatory disorders. Intracranial calcification especially in basal ganglia, interstitial lung disease, and early onset of the skin vasculopathy with chilblains and livedo reticularis are the most represented common features (4). The treatment is also distinctive and challenging. Furthermore, so far there is no consensus on the treatment of this type of disease. More and more studies found that IFNopathies poorly respond to conventional immunosuppressive treatments (5, 6). Thus, highly efficacious drugs are urgently needed.

JAK inhibitors (jakinibs) block the activation of the IFN pathway by inhibiting JAKs, resulting in a very promising therapeutic strategy for adults suffering from autoimmune, inflammatory, and hematological pathologies such as rheumatoid arthritis, psoriasis arthritis, and ulcerative colitis (7). In light of the pathogenesis of IFNopathies, the inhibition of the JAK activity shed light on a novel treatment target in these disorders. Indeed, several studies mostly in patients with SAVI and chronic atypical neutrophilic dermatitis with lipodystrophy (CANDLE) revealed that jakinibs improve the symptoms, control the disease activity, and suppress the IFN signature in most IFNopathy genotypes (8). However, the long-term outcome remains to be evaluated. Therefore, in this work, the outcomes of the treatment with jakinibs of 6 IFNopathy patients from a single center in China including 2 patients with SAVI, 2 patients with SPENCD, 1 patient with AGS1, and 1patient with AGS7 were reported.

METHODS

Patient Cohort and Study Approval

A retrospective analysis was performed on 6 patients who suffered from genetically confirmed IFNopathies between November 2017 and November 2021 in the Department of Pediatrics in Peking Union Medical College Hospital. All diagnoses were made by next-generation sequencing (NGS) and validated by Sanger sequencing. Age, gender, clinical manifestation, disease course, treatment, side effects, and laboratory data were collected from the electronic database. Some routine tests were performed at each follow-up visit including blood count, liver and kidney function tests, urine routine analysis, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), immunoglobulin level, complement, lymphocyte subset panels, and autoantibody tests. Chest high-resolution computed tomography, brain magnetic resonance imaging (MRI), cranial computerized tomography, plain X-ray, pulmonary function tests (PFTs), and 6-min walking test were also carried out every 6 or 12 months, depending on each patient, to evaluate the disease conditions. The study was approved by the Peking Union Medical College Hospital ethics committee and performed in accordance with the Declaration of Helsinki. Written informed parental consent was obtained for the use of jakinibs in the presence of a lawyer, and the consent of the parents was obtained for conducting the experiments.

Disease Activity Score

Disease activity rating scale of SAVI patients was evaluated according to the method described by Crow et al. (9), was used to evaluate the disease activity, and was determined at baseline as well as at each visit. The disease activity score of AGS patients was assessed by the AGS scale according to the method described by Adang (10) at each visit or based on the parental recall.

IFN Signature Assessment

The peripheral blood was collected into EDTA tubes. Total RNA was then extracted from the whole blood by RNA iso Plus (TaKaRa, Japan) following the manufacturer's instructions. RNA concentration was assessed by a spectrophotometer (Thermo Nanodrop 2000, USA). cDNA was derived from 200 ng total RNA and then quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in duplicate in 96-well plate using a SYBR Green Master Mix kit (Applied Biosystems, USA) and ABI7500 PCR system (Applied Biosystems, San Francisco, CA, USA). We studied 6 ISGs in the blood (IFIT1, IFI27, IFI44L, ISG15, SIGLEC1, RSAD2) as previously described (11). The relative expression was

calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to the geometric mean of the expression of 2 housekeeping genes: β-actin and OAZ. The sequence of the primers is listed in **Supplementary Table S1**. For each of the 6 ISGs, individual data were expressed relative to a single calibrator (pool of 28 healthy controls). The median fold change of the 6 ISGs was determined as an interferon score (IS) for each patient. An abnormal IS was defined as greater than +2 standard deviations above the mean of the control group (>2.56). IS was assessed before jakinibs treatment as well as at each follow-up visit.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 8.0.1, GraphPad Inc.). Data were expressed as median (minimum-maximum range). Unpaired *t*-test and one-way ANOVA were used to compare two or more groups, respectively. A value of $p < 0.05$ was considered statistically significant.

RESULTS

A total of 6 patients were included in our study: 2 patients with SAVI, 2 patients with SPENCD, 1 patient with AGS1, and 1 patient with AGS7. Among them, 3 were women. The median age of the disease onset was 1.8 years old (0.3–12.7 years old). The mean interval between the disease onset and the final genetic diagnosis was 5.6 years (0.1-12.3 years) and the mean interval between the disease onset and the start of the treatment with jakinibs was 6.6 years (1.1–13.5 years). The mean duration of the treatment was 2.5 years (1.25–4 years) (**Table 1**).

Clinical Manifestations

The most common features were febrile attacks and chilblain-like lesions which could be seen in 4 out of 6 patients (66.7%), respectively (**Figures 1A1, B1, C1**). Patient 2 also showed angiotelectasis in the back, and livedo reticularis was found in patient 4 who suffered from repeated ulcers and erythematous lesions in the cheeks and the arms as well (12) (**Figures 1C1, C2**). Failure to thrive is another universal characteristic. Patients 1 and 2 were below −2 standard deviation (SD) for weight; patient 6 was below the −2SD for height; and patient 4 was below the −2SD for weight, height, and head circumference. Patients 4 and 6 manifested with motor development delay, and patient 4 also showed language development delay. Other neurological features included congenital impairment in patient 3 as evidenced by narcolepsy, reduced memory, calculation, concentration, and academic performance, and limp in patients 3, 5, and 6. Four patients experienced infection susceptibility. Recurrent pneumonia occurred in patients 1 and 2. Patient 3 experienced recurrent respiratory tract infections at the early age of childhood, and *Staphylococcus aureus* and *Candida albicans* infection occurred in patient 4 who manifested severe skin vasculopathy. Other features included right ventricular enlargement (patient 2), glaucoma (patient 3), recurrent encephalalgia, and IgA nephropathy (patient 5). Patient 6 was initially diagnosed with autoimmune hepatitis according to the liver dysfunction, positive liver-kidney microsomal antibody, and liver puncture biopsy revealing moderate-to-severe interface hepatitis (**Table 1**).

Both SAVI patients were subjected to respiratory symptoms from the disease onset including cough, tachypnea, dyspnea, hypoxemia, and digital clubbing (**Table 1**). Patient 2 also presented with wheezing, crackles, cyanosis, and pulmonary hypertension (PH) due to the severe lung disease; thus, a lung biopsy was performed before the genetic diagnosis, revealing dilated alveolar cavity with a large number of foamy histiocytes, type II epithelial cell hyperplasia, widened alveolar septa, and fibrous tissue hyperplasia. In addition, right heart catheterization indicated precapillary PH. PFTs of patient 2 was also performed. His forced expiratory volume in 1 s was 31.6% predicted, and his diffusing capacity of the lungs for carbon monoxide was 24.1% predicted, which revealed severe restrictive lung function defect with reduced diffusion capacity.

Laboratory Investigations

Laboratory parameters showed elevated CRP in 2 patients and elevated ESR in 4 patients. Decreased white blood cell and neutrophil were present in 2 patients. Anemia was found in 1 patient, and lymphopenia was detected in 2 patients. The urine routine analysis revealed proteinuria and hematuria in 1 patient. Hypergammaglobulinemia was found in 4 patients. Complement 3 decreased in 2 patients. Alanine aminotransferase and aspartate transaminase were increased in 4 patients. Positive expression of antinuclear antibodies, antidouble-stranded DNA, antineutrophil cytoplasmic antibodies, rheumatoid factors, anticyclic citrullinated peptide, antihistone antibodies, and antiribonucleoprotein was found in 5 patients. Thyroid function tests revealed an increased thyroid-stimulating hormone in 3 patients (**Table 2**).

Imaging Examinations

Intracranial calcification was the predominant feature in IFNopathy patients. The symmetrical calcification of the basal ganglia was found in 4 patients, but not in the 2 SAVI patients (**Figures 1B2, C3, D1, D3** and **Supplementary Figures S1A–D**). In addition to intracranial calcification, patient 6 showed multiple liver calcification (**Figure 1E**). Patient 3 also presented with cerebral infarction in the right head of the caudate nucleus, right anterior limb of the internal capsule, and stenosis of the posterior cerebral artery (**Figures 1B3, B4**). Patient 5 presented middle cerebral artery occlusion as well (**Figure 1D2**). Leukodystrophy could be seen in 3 patients (**Figure 1C4**). Plain X-ray revealed the presence of platyspondyly and metaphyseal dysplasia in both patients 5 and 6 (**Supplementary Figures S1E, F**). Chest CT scan in patient 1 revealed diffuse cords, patchy consolidation, and ground-glass opacities (**Figure 1A2**). Multiple cords, reticular opacities, ground-glass opacities, and cysts were found predominantly in the lower lobes of patient 2 (**Figure 1A3**). Mild bilateral interstitial lung disease also occurred in patients 4 and 6 (**Supplementary Figures S2A, B**).

TABLE 1 | Clinical manifestations, treatment of IFNopathies patients.

	Patient 1	Patent 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex	M	M	F	M	F	F
Current age	3 years and 4 months	18 years	16 years and 9 months	5 years and 7 months	14 years and 7 months	17 years and 5 months
Age of onset	3 months	6 months	3 years	6 months	4 years	12 years and 8 months
Age of diagnosis	1 year and 3 months	12 years and 10 months	13 years and 4 months	2 years and 6 months	11 years and 11 months	13 years
Age of jakinibs treatment	1 years and 4 months	14 years	13 years and 4 months	3 years and 1 month	13 years and 4 months	15 years and 7 months
Mutation	TMEM173: p. N154S	TMEM173: p.V155M	TREX1: p. G47S; p.C154Mfs*3	IFIH1: p. A339D	ACP5: p. G215R; p. L247P	ACP5: p. S267Lfs*20; p. G239D
Diagnosis	SAVI	SAVI	AGS1	AGS7	SPENCD	SPENCD
Systemic inflammation	+	+	+	+	–	+
Febrile attacks	+	–	+	+	–	+
Failure to thrive	Wt < –2SD	Wt < –2SD	–	Wt < –2SD, Ht < –2SD, Hc < –2SD	–	Ht < –2SD
Infections	Pneumonia	Pneumonia	Cytomegalovirus, recurrent respiratory tract infections	*Staphylococcus aureus*, *Candida albicans*	–	–
Cutaneous manifestations	Chilblain-like lesions, ulcers, erythematous rashes	Chilblain-like lesions, angiotelectasis	Chilblain-like lesions, erythematous rashes	Chilblain-like lesions, erythematous rashes, ulcers, livedo reticularis	–	–
Neurological manifestations	–	–	Basal ganglia calcification, brain infarction, cognitive impairment, extrapyramidal symptoms	Basal ganglia calcification, leukodystrophy	Encephalalgia, intracranial calcification, leukodystrophy, middle cerebral artery occlusion, extrapyramidal symptoms	Intracranial calcification, leukodystrophy, extrapyramidal symptoms
Respiratory manifestations	Cough, tachypnea, dyspnea, hypoxemia, digital clubbing	Cough, tachypnea, dyspnea, hypoxemia, wheezing, crackles, cyanosis, digital clubbing, pulmonary arterial hypertension	–	–	–	–
Interstitial lung disease	+	+	–	+	–	+
Skeletal manifestations	–	–	–	–	Platyspondyly, metaphyseal dysplasia	Short stature, platyspondyly, metaphyseal dysplasia
Other features	–	Right ventricular enlargement	Glaucoma	Hypothyroidism	IgA nephropathy	Liver calcifications, hypothyroidism, AIH
Treatment before jakinibs	Prednisone	Ambrisentan, tadalafil	–	Methylprednisolone, IVIG	–	Prednisone, hydroxychloroquine, levothyroxine sodium, IVIG, MMF
Jakinibs	Tofacitinib (0.50 mg/kg/day), ruxolitinib (0.50 mg/kg/day)	Tofacitinib (0.38 mg/kg/day)	Ruxolitinib (0.25 mg/kg/day)	Ruxolitinib (0.71 mg/kg/day)	Tofacitinib (0.24 mg/kg/day)	Tofacitinib (0.22 mg/kg/day)
Concomitant treatment	Prednisone, thalidomide	Prednisone, ambrisentan, tadalafil, theophylline, sulfamethoxazole	Prednisone, thalidomide, aspirin	Prednisone, thalidomide, levothyroxine sodium	–	Prednisone, hydroxychloroquine, levothyroxine sodium

M, male; F, female; SAVI, STING-associated vasculopathy with onset in infancy; AGS, Aicardi–Goutières syndrome; SPENCD, spondyloenchondrodysplasia with immune dysregulation; Wt, weight; Ht, height; SD, standard deviation; IVIG, intravenous immunoglobulin; +, presence; –, absence.

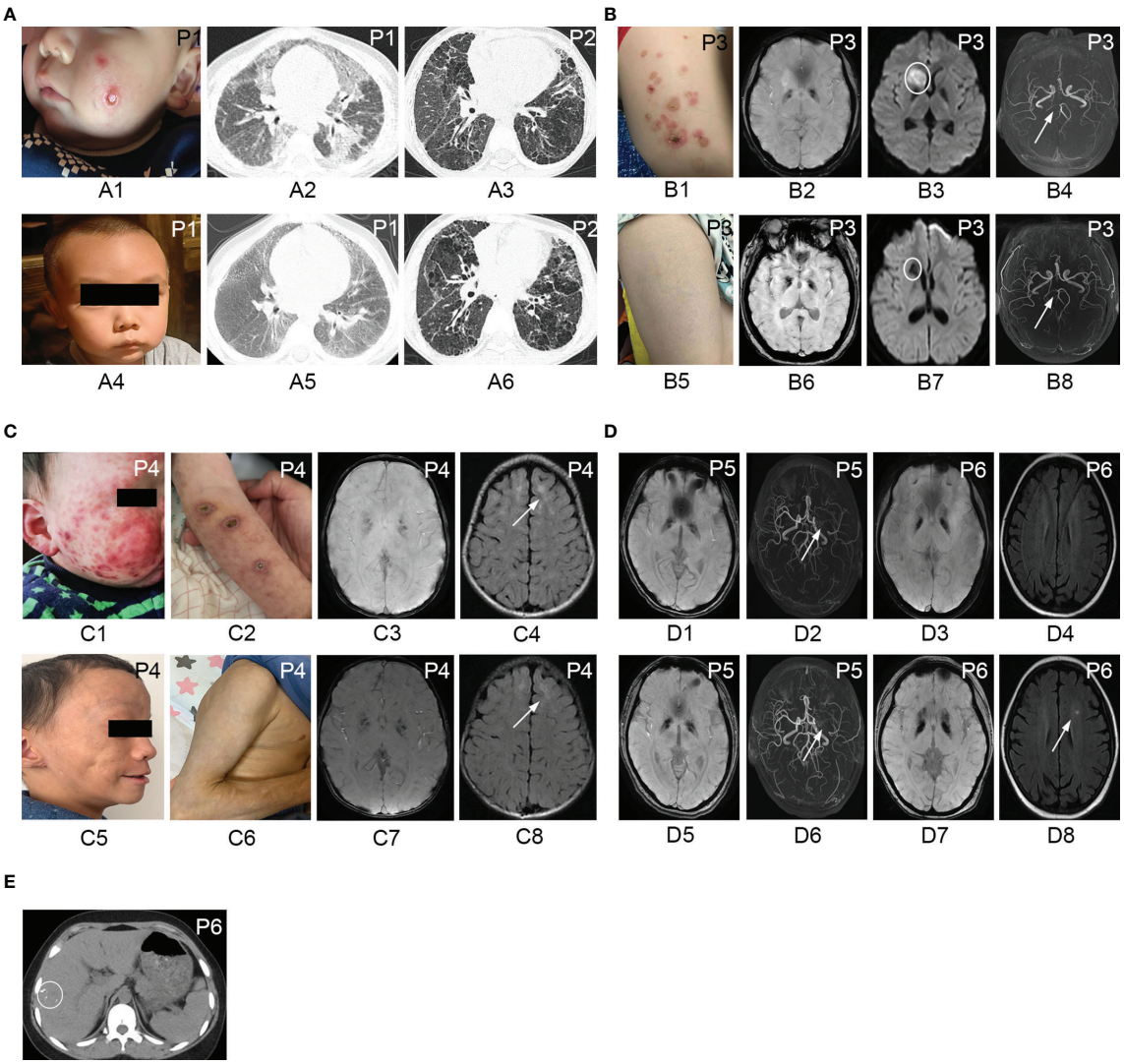

FIGURE 1 | Cutaneous manifestations, chest CT, and brain MRI of patients with IFNopathies before and after the treatment with jakinibs. Chilblain-like lesions and ulcerations on the cheek of patient 1 **(A1)** disappeared after the treatment with ruxolitinib **(A4)**. Chest CT scan of patient 1 demonstrating diffuse cords, patchy consolidation, and ground-glass opacities **(A2)** before the treatment, which improved after the treatment **(A5)**. Chest CT scan showing no significant improvement of reticular opacities, ground-glass opacities, and cysts in patient 2 before **(A3)** and after **(A6)** the treatment with tofacitinib. Erythematous rashes on the left leg of patient 3 **(B1)** resolved after the treatment with ruxolitinib **(B5)**. Susceptibility weighted imaging showing symmetric hypointense signal in the basal ganglia region in patient 3 **(B2)**, patient 4 **(C3)**, patient 5 **(D1)**, and patient 6 **(D3)** corresponding to calcification areas in brain CT scan and no improvement observed after the treatment **(B6, C7, D5, D7)**. Diffusion weighted imaging showing hyperintensity in the right head of the caudate nucleus and right anterior limb of the internal capsule **(B3)** in patient 3. Repeated MRI showing hypointense signal in diffusion weighted imaging **(B7)** indicating encephalomalacia after the treatment. Brain magnetic resonance angiography indicating stenosis and occlusion of the posterior cerebral artery **(B4)** in patient 3 and recanalization after the treatment **(B8)**. Erythematous rashes, chilblain-like lesions, ulcerations on the cheeks, and the arm of patient 4 **(C1, C2)** disappeared after the treatment with ruxolitinib, left with subcutaneous lipoatrophy **(C5, C6)**. T2 FLAIR MRI showing hyperintensity in the bilateral frontal lobe in patient 4 **(C4)** and no improvement after the treatment with ruxolitinib **(C8)**. Brain magnetic resonance angiography reveling occlusion of the left middle cerebral artery with the formation of peripheral collateral circulation with no changes before **(D2)** and after the treatment **(D6)** in patient 5. T2-weighted MRI of patient 6 demonstrating hyperintensity in the left corona radiata **(D8)** the after treatment with tofacitinib which was not observed before the treatment **(D4)**. Abdominal CT scan showing multiple calcifications in the liver in patient 6 **(E)**. P, patient.

ISG and IS Analysis

ISG expression was evaluated in 5 patients prior to jakinib treatment, and all of them showed a dramatically increased IS with a median of 29.62 (21.42–135.54) compared with healthy controls ($p < 0.0001$).

TREATMENT AND OUTCOMES

Treatment

Patients 1, 2, 5, and 6 received tofacitinib with a median dosage of 0.26 (0.12–0.3) mg/kg/day at start, and the dosage was gradually

TABLE 2 | Laboratory parameter changes before and after jakinibs treatment.

	Patient 1		Patient 2		Patient 3		Patient 4		Patient 5		Patient 6	
	M0	Mmax	M0	Mmax	M0	Mmax	M0	Mmax	M0	Mmax	M0	Mmax
Blood count	N	WBC, NEU#, ALC↓	N	ALC↓	N	N	WBC, NEU#↓	N	N	NEU#, ALC↓	WBC, NEU#, ALC, HgB↓	WBC, NEU#, ALC, HgB↓
CRP (mg/L)	13	10	12	14	2	5	4	1	1	1	1	2
ESR (mm/h)	28	21	78	84	25	6	44	15	6	8	19	32
Urine routine	N	N	N	N	Proteinuria, hematuria	N	N	N	N	N	N	Proteinuria
Liver function	N	N	N	N	ALT, AST↑	N	ALT, AST↑	N	N	N	ALT, AST↑	N
IgG (g/L)	13.08↑	18.07↑	22.16↑	29.07↑	17.46↑	9.87	24.27↑	17.49↑	14.19	14.94	8.72	13.66
IgA (g/L)	0.9	3.63↑	4.73↑	6.46↑	2.68↑	1.72	4.73↑	4.51↑	4.48↑	5.02↑	4.17↑	5.79↑
IgM (g/L)	1.19	1.07	0.72	0.71	0.75	0.34↓	0.56	0.73	0.82	0.77	0.16↓	0.24↓
Complement	C3↓	N	N	N	C3↓	N	N	N	N	N	N	N
Lymphocytes (/μl)	3,920	980↓	2,812	1,320↓	NA	NA	2,030↓	NA	3,001	1,420↓	263↓	341↓
CD3+ lymphocytes (/μl)	3,066	662↓	2,078	810↓	NA	NA	1,624↓	NA	1,651	723↓	198↓	287↓
CD3+CD4 +lymphocytes (/μl)	976	160↓	793	269↓	NA	NA	646↓	NA	684	293↓	90↓	129↓
CD3+CD8 +lymphocytes (/μl)	2,011	476↓	1,234	536	NA	NA	924	NA	786	352↓	98↓	144↓
B cells (/μl)	710	268↓	574↑	261	NA	NA	35↓	NA	1,077↑	656	19↓	39↓
NK cells (/μl)	63↓	67↓	152↓	285	NA	NA	309	NA	237	24↓	43↓	12↓
Autoantibodies	ANA, dsDNA, ANCA, AHA, CCP, RF	ANA	RF	N	ANA, dsDNA	ANA	N	N	RNP, RF	AHA, RF	ANA	ANA, Coombs
Thyroid function	NA	NA	N	N	N	N	TSH↑	N	TSH↑	N	TSH↑	TSH↑
Interferon score	21.42	32.3	NA	17.41	NA	4.95	23.11	21.79	135.54	19.67	36.13	19.46

WBC, white blood count; NEU#, neutrophil; HgB, hemoglobin; ALC, absolute lymphocyte count; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; ALT, alanine transaminase; AST, aspartate transaminase; Ig, immunoglobulin; ANA, antinuclear antibodies; dsDNA, antidouble stranded DNA; AHA, antihistone antibodies; CCP, anticyclic citrullinated peptide; RF, rheumatoid factors; RNP, antiribonucleoprotein; TSH, thyroid-stimulating hormone; ↑, higher than the normal range; ↓, lower than the normal range; M0, the time before jakinibs treatment; Mmax, time at the last follow-up visit; N, normal; NA, not available.

escalated according to the treatment response. Patient 5, who predominantly suffered from headache and extrapyramidal symptoms without detectable systemic inflammation all the time, was also treated with jakinibs considering the neurological manifestations and the dramatically increased IS. Patients 3 and 4 were subjected to ruxolitinib treatment with a dosage of 5 mg twice daily and 2.5 mg twice daily, respectively. The choice of jakinibs depended on the availability of drugs and the affordability of the patients. Tofacitinib was replaced with ruxolitinib in patient 1 after 15 months due to the unsatisfied control of CRP and ESR. All patients were also subjected to a treatment with a combination of corticosteroids, with the exception of patient 5 who never demonstrated any systemic inflammation. Although the cutaneous lesions and febrile attacks were considerably controlled, ESR was a consequence of the systemic inflammation that remained increased in patients 1, 3, and, and the dosage of jakinibs was already high enough. In addition, considering the side effects in the long-term use of corticosteroids, a concomitant treatment with thalidomide was prescribed for patients 1, 3, and 4 for the suppression of the systemic inflammation. Patient 2 also received anti-PH treatment. Other treatments included aspirin (patient 3), levothyroxine sodium (patients 4 and 6), and hydroxychloroquine (patient 6) (**Table 1** and **Figure 2A**).

Improvement of Clinical Symptoms After Treatment With Jakinibs

Febrile attacks and cutaneous lesions subsided in all patients after treatment with jakinibs (**Figures 1A4, B5, C5, C6, 2B**). Of note, the most prompt improvement of rashes was observed within 1 month after the start of jakinibs. The rashes completely disappeared in patients 2 and 3 and appeared only in winter in patients 1 and 4 with less severity and duration. However, subcutaneous lipoatrophy remained after remission of rashes in patient 4. Patients 2 and 3 were completely weaned from the treatment of corticosteroid. The median dosage of prednisone dropped to 0.20 (0.11–0.36) mg/kg/day in the remaining 3 patients. Four patients who had growth failure at baseline also got some improvement, although not statistically significant. Catch-up with growth was observed in patient 2 (−1.12 SD to −0.54 SD), patient 4 (−2.73 SD to −1.54 SD), and patient 6 (−4.48 SD to −4.08 SD). Weight gain was observed in patient 1 (−3.13 SD to −1.7 SD) and patient 6 (−1.48 SD to −1.26 SD) (**Figures 3A, B**). The normalization of head circumference (−3.15 SD to −1 SD) was also observed in patient 4. However, patient 1 developed further insufficiency for height, patients 2 and 4 developed further insufficiency for weight. The limp of 3 patients remained stable, and no extra neurological symptoms developed during the treatment.

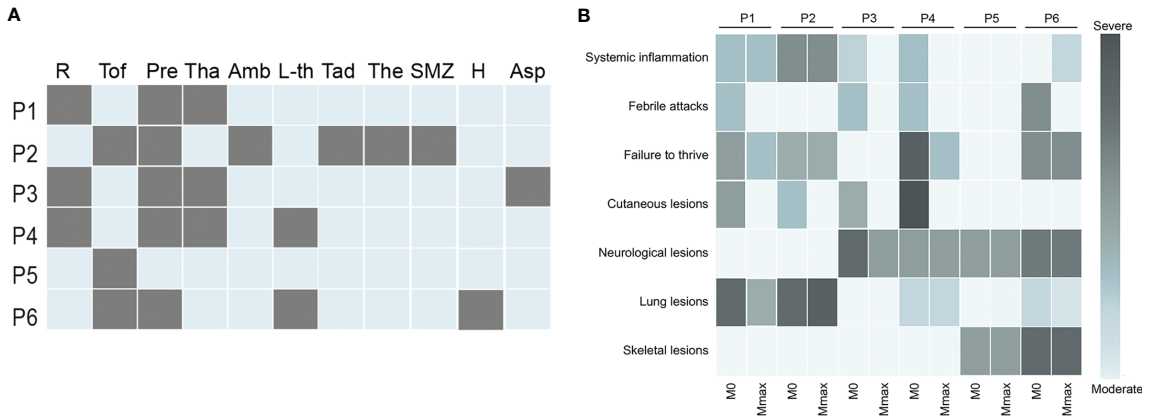

FIGURE 2 | Treatment and prognosis of patients with IFNopathies. **(A)** The treatment of patients with IFNopathies. Each row represents a patient, and each column represents a drug. A grey box indicates the presence of this treatment. **(B)** The change of clinical manifestations before and after jakinib treatment. Each row represents a manifestation, and each column represents a patient. The shade of color indicates the severity of the symptoms. The light blue box represents the absence of this symptom. P, patient; R, ruxolitinib; Tof, tofacitinib; Pre, prednisone; Tha, thalidomide; Amb, ambrisentan; L-th, levothyroxine sodium; Tad, tadalafil; The, theophylline; SMZ, sulfamethoxazole; H, hydroxychloroquine; Asp, aspirin; M0, the time before jakinibs treatment; Mmax, the time at the last follow-up visit.

The disease score of 2 patients with SAVI was also significantly reduced ($p < 0.05$) (**Figure 3C**). Respiratory symptoms alleviated significantly with a considerable amelioration of cough, dyspnea, cyanosis, and tachypnea, and the daily activities became largely limitless for both patients. The digital clubbing of patient 1 also achieved significant remission, and no digital loss occurred. Repeated right heart catheterization in patient 2 resulted in a reduction of PH from 57 mmHg at baseline to 32 mmHg at the last visit. His forced expiratory volume in 1 s on PFTs improved from 31.6% predicted at baseline to 35% predicted after the treatment and the diffusing capacity of the lungs for carbon monoxide remained stable to 24% predicted. The 6-min walking test remained stable with a distance of 510 m. In addition to the amelioration of fevers and rashes, the AGS scale of patients 3 and 4 experienced an elevation to full score and kept stable in the following visit (**Figure 3D**). Patient 3, who showed loss of ambulation and severe cognitive impairment as evidenced by narcolepsy, as well as reduced memory, calculation, and concentration, recovered rapidly and sustainably at 1 week after ruxolitinib treatment. The gross motor and language development of patient 4 also improved. Patient 5 no longer experienced headaches after the treatment with tofacitinib.

Heterogeneous Improvement of Laboratory Parameters and IS

No significant change in CRP levels was observed in all patients. It fluctuated during the treatment and remained high in 2 patients at the last visit. In patients with normal CRP levels at baseline had no significant upward trend (**Figure 3E**). While the reduction of ESR was not significant after the treatment (**Figure 3F**), patients 3 and 4 finally got a normalization of ESR after the treatment with ruxolitinib. Nevertheless, patient 6 whose ESR level was normal at baseline experienced an increase of ESR at the last visit without any complaints and detected infections. Blood count improved in patient 4 and was reduced in 3 patients. Patient 6 with anemic and lymphopenia at baseline did not experience any increase after the treatment. Proteinuria and hematuria were also quickly under control in patient 3, while patient 6 developed proteinuria during the last visit. Liver function continuously improved with the treatment, reaching a normal range in all patients. C3 levels also returned to normal. Hypergammaglobulinemia did not show any improvement. Autoantibodies were reduced in patients 1, 2, and 3 (**Table 2**). However, a positive Coombs test was found in patient 6. IS also significantly decreased ($p < 0.01$) during the treatment but remained elevated (**Figure 3G**).

Stable of Imaging Examinations After Jakinib Treatment

The repeated chest CT scan of 4 patients revealed a remarkable improvement in patient 1 (**Figure 1A5**), and no noticeable change was observed in patient 2 (**Figure 1A6**), patient 4, and patient 6 (**Supplementary Figures S2D, E**). Bilateral basal ganglia calcifications found in 4 patients at baseline were not reduced in any of the repeated brain MRI (**Figures 1B6, C7, D5, D7**). Enephalomalacia on MRI was also reduced, and stenosis of the cerebral artery disappeared completely in patient 3 (**Figures 1B7, B8** and **Supplementary Figures S2C, F**). Hyperintensity in the bilateral frontal lobe indicating leukodystrophy in patient 4 remained existent (**Figure 1C8**). The occlusion of the left middle cerebral artery in patient 5 still existed (**Figure 1D6**). However, T2-weighted MRI in patient 6 demonstrated new hyperintensity, which was not observed before (**Figure 1D4**), in the left corona radiata after the treatment with tofacitinib (**Figure 1D8**).

Side Effects

Overall, tofacitinib and ruxolitinib were well tolerated in all patients. No severe side effects were documented, and no deaths were reported. Only a transient lung infection was reported in patient 1. Patient 3 suffered from cytomegalovirus

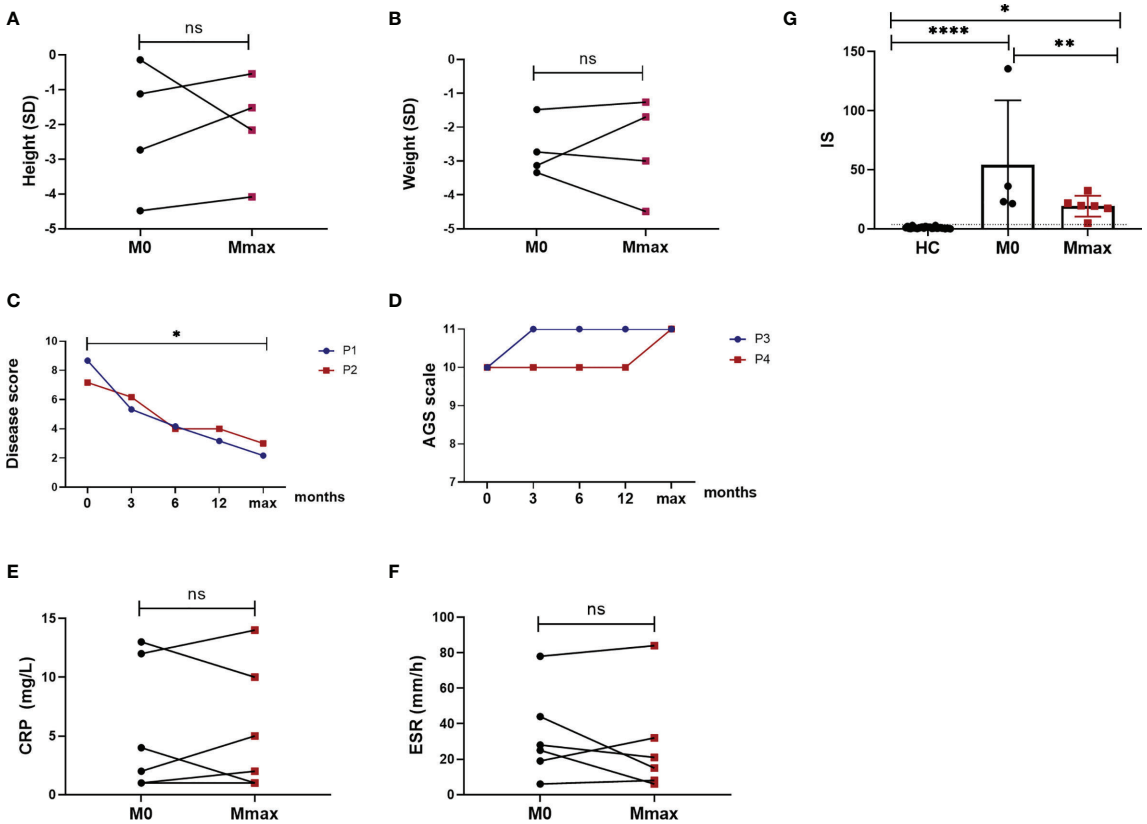

FIGURE 3 | Response to jakinib treatment. Height in standard deviation (SD) **(A)** showing catch-up growth in 3 patients and further failure to thrive in 1 patient (ns). Weight **(B)** in SD showing weight gain in 2 patients and further growth failure in 2 patients (ns). Disease score of 2 patients with SAVI improved after the treatment ($p < 0.05$) **(C)**. AGS scale elevated and stayed stable during the treatment in patient 3 and patient 4 **(D)**. Measures of CRP **(E)** and ESR **(F)** showing no improvement after the treatment (ns). Six gene-based IFN score **(G)** decreased after the treatment ($p < 0.01$). The dotted line indicates the cutoff value (2.56). HC, healthy control; M0, the time before jakinib treatment; Mmax, the time at the last follow-up visit; P, patient; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; ns, nonsignificant.

infection at the beginning of ruxolitinib treatment, but this infection did not aggravate further after the treatment. Although 4 patients developed lymphopenia, which could predispose them to infections, there were no infections detected on regular screening during the treatment.

DISCUSSION

IFNopathies are a group of rare inherited autoinflammatory diseases characterized by a constitutive overproduction of type I IFN, which is due primarily by defects in the genes related to nuclear sensing, metabolism, and negative regulation of type I IFN pathway. These IFNopathies include the well-known AGS, SAVI, and CANDLE (13). Ten years have passed since the term "IFNopathies" was coined. It is suggested that as many as 38 genetic defects are currently responsible for this group of diseases (14). Timely diagnosis and treatment are critical for patients' prognosis due to the high morbidity and mortality of these diseases. However, most of these diseases are resistant to conventional immunosuppressive therapy. Jakinibs, including tofacitinib, a blocker of JAK 1/3, and ruxolitinib and baricitinib, blockers of JAK 1/2, have recently shown favorable results in the treatment of these types of diseases (5, 8, 10, 15). Our study further confirmed that jakinibs could be considered a promising therapeutic option for IFNopathies not only for SAVI and CANDLE but also for AGS and SPENCD. Our results demonstrated that the treatment with jakinibs, tofacitinib or ruxolitinib, reduced disease flare-ups and improved symptoms. All patients experienced a significant improvement in cutaneous lesions except patients 5 and 6 who never developed rashes (**Figure 2B**). Three patients were able to permanently free from corticosteroid treatment without disease aggravation. Of note, patient 4 was left with lipoatrophy after the rashes subsided (**Figures 1C5, C6**). Four patients with recurrent episodes of fever completely normalized their body temperature. Intriguingly, patient 2 developed fever again after taking prednisone, and his temperature improved after the withdrawal from it, which was never reported in previous studies. Failure to thrive, another striking feature of IFNopathies, showed heterogeneous improvement in our patients (**Figures 3A, B**).

For SAVI patients, both individuals got amelioration of disease activity. Patient 1, who changed the treatment from

tofacitinib to ruxolitinib after 15 months, experienced a dramatic improvement of the lung lesions and rashes after ruxolitinib started, suggesting that tofacitinib, to some extent, is not the best choice for patients with SAVI. Actually, a similar phenomenon was observed in a recent study where tofacitinib failed to halt disease deterioration in 3 patients with SAVI (16). Conversely, ruxolitinib showed considerable results in other studies (6, 9, 17). Although no significant improvement was observed in patient 2 under the concomitant treatment with tofacitinib with ambrisentan and tadalafil, no further exacerbation of the interstitial lung disease was observed, including the stabilization of PFTs, 6-min walking test, and improvement in PH. The variability of disease severity, inadequate plasma drug levels, organ impaired degree prior to jakinib treatment, and the genotype may also contribute to the heterogeneous improvement of this disease (17–19), suggesting that the treatment should be initiated as soon as possible to avoid an irreversible lung damage (20). Intracranial calcification is another universal imaging feature of IFNopathies, found in 4 of our patients. Moreover, patient 6 showed multiple liver calcifications, which were not reported before and might be associated with hepatocellular injury. Although the neurological symptoms improved, the calcifications continuously existed during jakinib treatment. The ability of the drug to penetrate the blood–brain barrier might play a pivotal role. Some studies have found that the concentration of ruxolitinib in the cerebral spinal fluid was only 10%–15% of that in the plasma (21, 22). This inability of the drugs to cross the blood–brain barrier should be taken into consideration when choosing the treatment of patients with neurological symptoms. Of note, the 2 patients with SPENCD in our study demonstrated different responses to the treatment with tofacitinib. Patient 5 achieved a considerable remission after the treatment. However, patient 6 experienced an unsatisfactory disease control as evidenced by increase of ESR, proteinuria, new cerebral lesions, and persistent lymphopenia, although the patients did not have any subjective complaint. Only 1 patient with SPENCD has been reported to date who received baricitinib treatment and achieved symptom control (23). Therefore, more studies are needed to assess the efficacy of jakinibs in patients with SPENCD.

Despite the significant improvement of disease signs and symptoms under the treatment of jakinibs, the systemic inflammatory markers (ESR and CRP) hardly returned to normal, in accordance with previous studies, indicating that many other cytokines besides type I IFN may also play a role in the development of the disease (5). This also suggests that a combined treatment is necessary for these patients. In our study, although no significant decrease in CRP and ESR was observed (**Figures 3E, F**), patients 3 and 4 finally reached the normalization of ESR. In addition to ruxolitinib and corticosteroid, both patients received a combined treatment with thalidomide, then CRP and ESR levels continuously decreased after. A decrease in CRP and ESR was also observed in patient 1 with the addition of thalidomide, albeit they remained above the normal levels. Our study suggests that thalidomide could be a recommended concomitant drug when inflammatory markers are consistently high. Recent studies found that thalidomide can reduce the level of cytokines and antiangiogenic factors and has a strong immunomodulatory effect. It is now widely used in rheumatoid arthritis, inflammatory bowel disease, and cancers, showing encouraging results (24). However, the number of patients in our study was too small and a larger cohort is needed to validate our results. On the other hand, the incomplete inhibition of the JAK-STAT pathway might also contribute to a suboptimal control of CRP and ESR levels (9). IS in our study decreased in most patients during the treatment with jakinibs, in line with previous studies, with a magnitude of the decrease varying from patient to patient and being more pronounced in patients with SPENCD. Although IS decreased, the absolute levels remained elevated (**Figure 3G**). On the contrary, patient 1 whose symptoms were well controlled, experienced an increase of IS in the last test. Several factors might lead to unparalleled symptom improvement and IS. The variable and incomplete reduction of IS could be explained by the transient inhibition of pSTAT1 caused by the rapid kinetic of JAK inhibition observed in *ex vitro* experiments (9). In addition, the evaluation of 6 gene-based assays is not enough because of the very small number of genes to fully reflect the activation of the IFN pathway, where hundreds of ISGs are induced, especially in different subtypes of IFNopathies, and also, no consensus exists on the groups of ISGs. In addition to the incomplete inhibition of the IFN pathway, changes in other cytokines and pathways, including IL-6, IL-1B, and NF-κB, were already observed in IFNopathies; thus, they might also lead to such a result (5, 19, 25).

Considering that jakinibs not only inhibit the IFN pathway but also have effects on other inflammatory signaling pathways such as IL-6 and IFN-γ, extra attention should be paid on the occurrence of side effects when using jakinibs (26). Several side effects have been documented in previous studies, with infections being the most remarkable. Upper respiratory tract infections are the most recorded. Sanchez et al. also observed polyomavirus viremia in patients during baricitinib treatment (15). Therefore, a regular screening of this virus should be considered, especially for SAVI patients whose underlying lung defect make them vulnerable to infections. Fortunately, the treatment with tofacitinib or ruxolitinib was overall well tolerated by our patients and no one developed severe infections during the treatment. Patient 3 who had a *cytomegalovirus* infection prior to ruxolitinib treatment did not further worsen after the start of the treatment. However, 3 patients in our study developed lymphopenia during the treatment, which was not reported in previous studies. Indeed, Sanchez et al. showed that the absolute lymphocyte count increased along with disease improvement after the treatment in patients with lymphopenia at baseline (15). In addition, considering the widespread effects of other cytokines, a cytokine rebound effect should not be ignored when the treatment is discontinued, as it was reported in a previous study (9).

Given the widespread influence on other cytokines, more selective inhibitors or other avenues are needed to avoid severe side effects. For instance, filgotinib and upadacitinib, both selective inhibitors of JAK1, are promising candidates in the future, as well as the monoclonal antibody to type I IFN receptor subunit 1 (IFNAR1) anifrolumab, which showed promising effects in systemic lupus erythematosus (27). Treatment with reverse transcriptase inhibitors also showed encouraging results in patients with AGS1-AGS5 in which the diseases are predominantly mediated *via* an RNA signaling pathway (28). Hematopoietic stem cell transplantation, widely used as a therapy able to cure other inborn errors of immunity has not been well studied in IFNopathies to date. Kataoka et al. recently reported a patient with PSMB9 mutation who was cured by hematopoietic stem cell transplantation (29). However, more studies are needed to confirm its efficacy in other IFNopathies, especially when severe neurological lesions or lung defects occur.

In conclusion, our results indicated that jakinibs including ruxolitinib and tofacitinib are promising therapies in patients with SAVI, AGS, and SPENCD, especially for cutaneous lesions and febrile attacks. Our results also demonstrated that the combined therapy is essential for these patients. Considering the heterogeneous improvement observed in previous studies, prospective studies are necessary, as well as an urgent need to explore other effective approaches.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Peking Union Medical College Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HS designed this study, supervised the study, and revised the manuscript. WL collected clinical data, followed the patients, performed experiments, and drafted the manuscript. WW (2nd author) contributed to the genetic analysis and supervised the study. WW (3rd author), LZ, LG, JM, CW, MQ, XT, YZ, SJ, LW, and MM followed the patients. All authors have read and approved the manuscript and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.825367/full#supplementary-material

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