frontiers | Research Topics

Community series in primary immunodeficiencies worldwide, volume II

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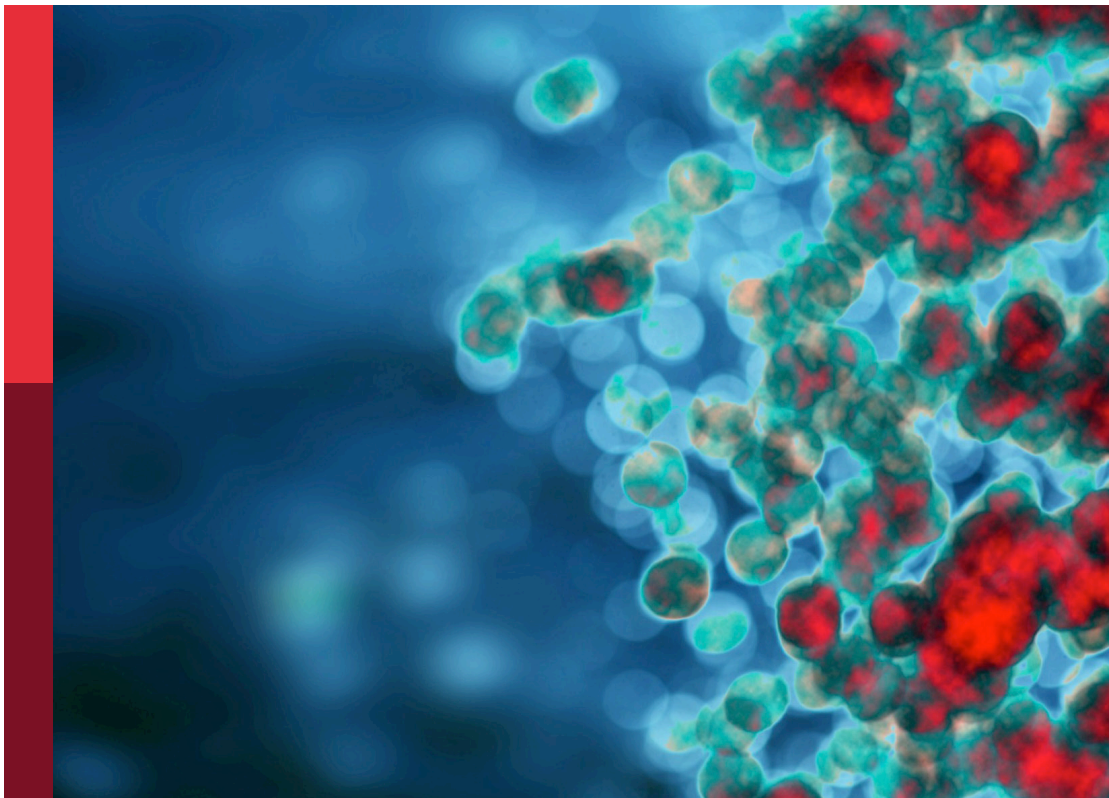
Published in
Frontiers in Immunology



ISSN 1664-8714
ISBN 978-2-8325-6063-1
DOI 10.3389/978-2-8325-6063-1

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Community series in primary immunodeficiencies worldwide, volume II

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Citation

Kanegane, H., Condino-Neto, A., Korganow, A.-S., eds. (2025). *Community series in primary immunodeficiencies worldwide, volume II*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6063-1

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 19 February 2025
DOI 10.3389/fimmu.2025.1564959

OPEN ACCESS

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RECEIVED 22 January 2025
ACCEPTED 05 February 2025
PUBLISHED 19 February 2025

CITATION
Condino-Neto A, Korganow A-S and Kanegane H (2025) Editorial: Community series in primary immunodeficiencies worldwide, volume II.
Front. Immunol. 16:1564959.
doi: 10.3389/fimmu.2025.1564959

Editorial: Community series in primary immunodeficiencies worldwide, volume II

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KEYWORDS

primary immunodefciencies, inborn errors in immunity, epidemiology, genetic screen, newborn screen (NBS)

Editorial on the Research Topic
Community series in primary immunodeficiencies worldwide, volume II

Primary immunodeficiencies (PIDs), also referred to as inborn errors of immunity, are rare but impactful conditions characterized by impaired immune function, leading to increased susceptibility to infection and immune dysregulation. This Research Topic summarizes the key findings from the second volume of the Research Topic Community Series, focusing on advances in genetic understanding, diagnostics, and management strategies.

Expanding the genetic and clinical landscape of PIDs

Research continues to uncover the genetic basis of PIDs, shedding light on their clinical variability. A case report by Chen et al. illustrates how mutations in the recombination-activating gene 1 (*RAG1*) can present atypically as autoimmune hemolytic anemia. By leveraging next-generation sequencing and lymphocyte subset analysis, the case underscores the need for genetic tools to identify atypical PID presentations.

Similarly, Jiang et al. report a novel mutation in the *IL2RG* gene linked to X-linked severe combined immunodeficiency (X-SCID). Despite intensive anti-infective treatment, the patient succumbed to disseminated BCG disease. This case highlights the critical role of early diagnosis and timely allogeneic hematopoietic cell transplantation (HCT), emphasizing the importance of newborn screening (NBS) to improve outcomes for rare PIDs such as X-SCID.

Newborn screening and early intervention

NBS has transformed the early detection of PIDs, enabling life-saving interventions. Beppu et al. document the successful diagnosis of X-SCID in Japan through NBS, which

enabled early HCT without complications. However, the global implementation of NBS faces challenges, such as limited infrastructure and disparities in healthcare access.

Äng et al. demonstrate the limitations of current TREC-based NBS algorithms, which fail to detect certain PIDs, such as *IKZF1*-related combined immunodeficiency. This underscores the importance of integrating kappa-deleting recombination excision circles into NBS protocols to capture a broader range of immune disorders.

Innovative diagnostic and screening tools

Cost-effective diagnostic tools remain essential in resource-limited settings. Sartorelli de Toledo Piza et al. propose calculated globulin as a viable screening alternative for antibody deficiencies, particularly hypogammaglobulinemia. Their findings suggest that calculated globulin has a strong correlation with IgG levels, thus bridging diagnostic gaps in underserved regions.

Flow cytometry (FCM), as demonstrated by Tahiat et al., offers a versatile and cost-effective diagnostic method for PIDs such as SCID, Omenn syndrome, and familial hemophagocytic lymphohistiocytosis. In addition to identifying hallmark immunodeficiencies, FCM provides clues for conditions such as hyper-IgE syndrome and *STAT1* gain-of-function mutations, highlighting its adaptability in diverse clinical environments.

Immune dysregulation and malignancy in PIDs

Immune dysregulation, a common feature of PIDs, is associated with a heightened risk of malignancy and organ damage. Graafen et al. explore the immune profiles of patients with ataxia telangiectasia (AT), revealing deficiencies in T- and NK-cell function. These findings suggest therapeutic potential in targeting cytokine pathways or enhancing T- and NK-cell functionality.

Boyarchuk et al. offer a comprehensive review of Nijmegen breakage syndrome, a disorder linked to DNA repair defects, immunodeficiency, and malignancy. Their 25 years of experience in Ukraine underscore the urgency of regular monitoring, early chemotherapy, and immunoglobulin replacement therapy to mitigate risks. Notably, malignancy remains the leading cause of death in Nijmegen breakage syndrome, necessitating advancements in molecular diagnostics and therapeutic interventions.

Rare and atypical presentations

The phenotypic variability of PIDs presents diagnostic challenges. Al-Saud et al. describe a mild case of *STK4* mutation-related immunodeficiency with severe T-cell lymphopenia, showcasing how functional analysis can delineate disease severity. Similarly, Fink et al. report on severe aplastic anemia associated with *STAT1* gain-of-function mutations, broadening the understanding of immune dysregulation's impact on hematologic health.

Future directions

Collectively, the articles included in this Research Topic highlight progress in PID research, particularly in genetic diagnostics, NBS, and cost-effective tools such as calculated globulin and FCM. They also underscore the need to manage immune dysregulation and malignancies in conditions such as AT and Nijmegen breakage syndrome. Expanding NBS programs to underserved regions is crucial for reducing diagnostic delays.

Moreover, advancements in gene therapy and targeted biologics hold promise for transforming PID management. International collaboration will be vital in fostering equitable access to these innovations. For example, Sartorelli de Toledo Piza et al. illustrate how calculated globulin complements traditional IgG assessment, and Tahiat et al. demonstrate the diagnostic versatility of FCM.

Conclusion

The articles in this Research Topic highlight the complexity and diversity of PIDs, emphasizing the importance of early diagnosis, innovative diagnostic tools, and targeted therapies. By fostering global collaboration and sharing advancements, the PID community can advance the understanding and care for these conditions, ultimately improving outcomes for patients worldwide.

We extend our gratitude to the authors and contributors for their valuable insights and to the readers for their engagement with this Research Topic Community Series. This series plays a critical role in fostering an ongoing dialogue within the PID research community, providing a platform to share advancements, challenges, and innovative approaches to improve patient outcomes globally. Together, we strive for a future where no patient with a primary immunodeficiency is left undiagnosed or untreated.

Author contributions

AC-N: Writing – original draft, Writing – review & editing. A-SK: Writing – original draft, Writing – review & editing. HK: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 12 September 2023
DOI 10.3389/fimmu.2023.1257581

OPEN ACCESS

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RECEIVED 12 July 2023
ACCEPTED 04 August 2023
PUBLISHED 12 September 2023

CITATION
Äng C, Zetterström RH, Ramme K, Axelsen E, Marits P and Sundin M (2023) Case report: *IKZF1*-related early-onset CID is expected to be missed in TREC-based SCID screening but can be identified by determination of KREC levels. *Front. Immunol.* 14:1257581. doi: 10.3389/fimmu.2023.1257581

Case report: *IKZF1*-related early-onset CID is expected to be missed in TREC-based SCID screening but can be identified by determination of KREC levels

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This report illustrates a case that would have been missed in the most common screening algorithms used worldwide in newborn screening (NBS) for severe combined immunodeficiency (SCID). Our patient presented with a clinical picture that suggested a severe inborn error of immunity (IEI). The 6-month-old baby had normal T-cell receptor excision circle (TREC) levels but no measurable level of kappa-deleting recombination excision circles (KRECs) in the NBS sample. A *de novo IKZF1*-mutation (c.476A>G, p.Asn159Ser) was found. The clinical picture, immunologic workup, and genetic result were consistent with *IKZF1*-related combined immunodeficiency (CID). Our patient had symptomatic treatment and underwent allogeneic hematopoietic cell transplantation (HCT). *IKZF1*-related CID is a rare, serious, and early-onset disease; this case provides further insights into the phenotype, including KREC status.

KEYWORDS

SCID, IKZF1, Ikaros, newborn screening, CID, HCT (hematopoietic cell transplant)

Introduction

IEI are a diverse group of monogenic diseases characterized by a malfunctioning immune system. In recent years, screening for the most fatal form, SCID, has been implemented in various settings. Before the start of NBS for SCID, these patients commonly present with infections in their first 6 months of life, and because of their

10.3389/fimmu.2023.1257581

pronounced immunodeficiency, were unable to manage these infections. If left untreated, most succumbed within their first 2 years of life (1, 2). An early diagnosis has been associated with favorable outcome (3). Hence, in recent years NBS by measuring TREC levels using Guthrie cards has been implemented in many countries, including Sweden (4). TREC-based SCID NBS is expected to and has been demonstrated to identify other potential life-threatening T lymphopenic conditions, e.g., CID not fulfilling SCID criteria and thymic defects (5). The addition of KREC analysis in SCID NBS has been explored in research aiming at identifying serious CID and B cell immunodeficiency (6).

One IEI-associated gene is *IKZF1*. The IKZF1 protein is a member of the family of zinc-finger proteins (IKZF1-IKZF5) that bind DNA through four N-terminal zinc-finger domains and dimerize through two C-terminal zinc-fingers (7). It targets DNA sequences at pericentromeric heterochromatin regions of certain genes and regulates the nucleosome remodeling and histone deacetylase complex, and through that process either activates or represses transcription (8). IKZF1 is well-studied in its role as a regulator of hematopoiesis, with it being crucial for lymphocyte development, differentiation, and tumor suppression. Mutations in the gene have previously been reported to cause CID (9, 10).

We present a child with a *de novo* point mutation in *IKZF1*, resulting in a clinically severe form of early-onset CID. The child was born just before the Swedish introduction of SCID NBS but presented when national TREC-based NBS was up and running. Testing of the newborn sample at presentation was normal by TREC but pathologic by KREC levels.

Case description

At 6-month-old baby girl, born to non-consanguineous parents of Swedish descent (Caucasians), was admitted to a Swedish hospital due to severe pneumonia. Previous medical records showed failure-to-thrive and thrombocytosis. She received respiratory support, corticosteroids, and antibiotics at the intensive care unit. Rhino- and bocavirus were detected in nasal swabs, and radiology suggested a bacterial infection. Due to halting recovery after a week, extended testing was performed, revealing unmeasurable immunoglobulin levels and a nasopharyngeal swab PCR positive for *Pneumocystis jirovecii* (PCJ). Radiology and further microbiologic testing confirmed PCJ pneumonia. This raised a suspicion of SCID, and antimicrobial therapy was adjusted and intravenous immunoglobulin substitution initiated. The patient was transferred to a tertiary pediatric hospital for intensive care and immunologic workup and treatment. *Candida parapsiolosis* was found in the feces. The necessity of allogenic HCT was evident; thus, it was carried out. The timeline is depicted in Figure 1.

Diagnostic assessment

Upon clinical diagnosis, we retrieved the patient's NBS sample and ran an SCID NBS assay analyzing both TRECs and KRECs: the TREC copy number was 110 copies/well [recall threshold ≤5, population mean 79, IQR 55, 110 (4)] and KRECs were not measurable. Lymphocyte phenotyping in peripheral blood showed prevalent T lymphocytes of which the majority were $CD4^+$ T lymphocytes, with a smaller fraction of $CD8^+$ T lymphocytes. Both $CD4^+$ and $CD8^+$ T lymphocytes had a uniform, atypical surface phenotype expressing intermediate levels of the naïve cell marker CD45RA but high levels of the recent thymic emigrant marker CD31 (Table 1). Analysis of the T lymphocyte receptor (TCR) Vβ repertoire by flow cytometry revealed an essentially normal distribution in both $CD4^+$ and $CD8^+$ T lymphocytes, but an elevated level of CD4+ $V\beta2^+$ T lymphocytes (19%, upper normal limit 13%). B lymphocytes were severely reduced in numbers (0.02×10^9/L) and were all found to be naïve (93% with no plasmablasts and switched memory cells, Table 1). No specific antibodies to previous vaccinations were detected. Lymphocyte proliferation showed adequate response to mitogens, except for pokeweed mitogen (PWM) in B lymphocytes and concavalin A (ConA) in $CD8^+$ T lymphocytes. Conversely, a

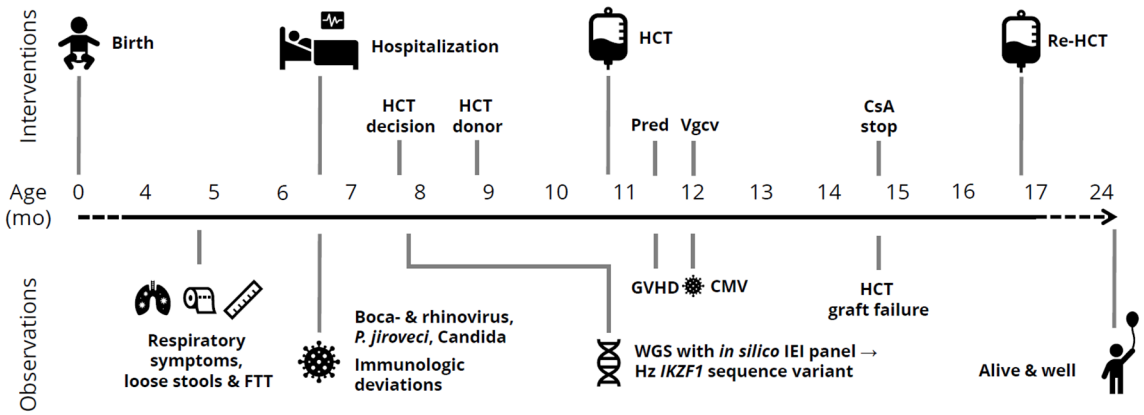

FIGURE 1
Timeline of clinical observations and interventions in a child with *IKZF1*-related CID. mo, months; FTT, failure-to-thrive; HCT, allogeneic hematopoietic cell transplantation; Pred, prednisolone; Vgcv, valganciclovir; GVHD, graft-versus-host disease; CMV, cytomegalovirus; IEI, inborn errors of immunity; Hz, heterozygous; CsA, ciclosporine A.

10.3389/fimmu.2023.1257581

TABLE 1 Hematologic and immunologic findings in a child with *IKZF1*-related CID.

	At diagnosis (6 mo old)	Post-HCT 1st (13 mo old)	Post-HCT 2nd (16 mo old)	Follow-up (22 mo old)
Full blood count				
Hemoglobin (g/L)	128	115	94 ↓	116
Leukocytes (10^9/L)	14.8	3.6 ↓	1.0 ↓	9.1
Neutrophils	6.4 ↑	3.3	0.9	4.2
Eosinophils	0 ↓	0.1	<0.1	0.1
Basophils	0.03	<0.1	<0.1	<0.1
Monocytes	0.5	<0.1 ↓	0.1 ↓	0.7
Lymphocytes	7.8 ↑	0.2 ↓	0.9 ↓	4.6
Thrombocytes (10^9/L)	720 ↑	246	271	327
T lymphocytes				
$CD3^+$ (10^9/L)	4.61	0.09 ↓	1.76	3.14
$CD4^+$ (10^9/L)	3.78	0.04 ↓	0.37 ↓	0.51 ↓
$CD8^+$ (10^9/L)	0.32 ↓	0.04 ↓	1.34	2.58
$CD4^+$ $CD45RA^+$ $CCR7^+$ (naïve, %)	>95 ↑ #	-	-	35
$CD4^+$ $CD45RA^+$ $CD31^+$ (RTE, %)	>95 ↑ #	–	–	–
$CD4^+$ $CD45RA^-$ $CCR7^-$ (EM, %)	↓ #	-	-	23
$CD4^+$ $CD45RA^-$ $CCR7^+$ (CM, %)	↓ #	–	–	35
$CD4^+$ $CD45RA^+$ $CCR7^-$ (EMRA, %)	↓ #	-	-	7
$CD8^+$ $CD45RA^-$ $CCR7^-$ (EM, %)	↓ #	–	–	25
$CD8^+$ $CD45RA^-$ $CCR7^+$ (CM, %)	↓ #	-	-	1
$CD8^+$ $CD45RA^+$ $CCR7^-$ (EMRA, %)	↓ #	–	–	72
Lymphocyte proliferation (counts/μL)				
CD4+ PWM	2145 ↑	–	–	–
CD8+ PWM	633 ↓	-	-	-
$CD19^+$ PWM	1 ↓	–	–	–
$CD4^+$ ConA	822	-	-	-
$CD8^+$ ConA	144 ↓	–	–	–
$CD4^+$ Pneumococcus	0 ↓	-	-	-
$CD8^+$ Pneumococcus	0 ↓	–	–	–
$CD4^+$ Tetanus toxin	0 ↓	-	-	-
$CD8^+$ Tetanus toxin	0 ↓	–	–	–
$CD4^+$ Candida	0 ↓	-	-	-
$CD8^+$ Candida	0 ↓	–	–	–
B lymphocytes				
$CD19^+$ (10^9/L)	0.02 ↓	0.01 ↓	0.01 ↓	0.13 ↓
$CD19^+$ IgD^+ $CD27^-$ (naïve, %)	93 ↑	-	-	-
$CD19^+$ IgD^- $CD27^+$ (SM, %)	<0.5 ↓	–	–	–

(Continued)

10.3389/fimmu.2023.1257581

TABLE 1 Continued

	At diagnosis (6 mo old)	Post-HCT 1st (13 mo old)	Post-HCT 2nd (16 mo old)	Follow-up (22 mo old)
NK cells				
$CD16^+$ $CD56^+$ (10^9/L)	0.07 ↓	0.14 ↓	0.12	0.12
Immunoglobulin levels				
IgG (g/L)	<0.1 ↓	3.8	5.9	7.4
IgA (g/L)	<0.08 ↓	<0.08 ↓	0.24	0.27
IgM (g/L)	<0.1 ↓	<0.1 ↓	0.13 ↓	0.35
IgE (IU/L)	<2.00 ↓	–	–	–
Pneumococcus IgG (mg/L)	<0.01 ↓	–	–	–
Tetanus IgG (IU/mL)	<0.1 ↓	-	-	-
Chimerism (% recipient)				
$CD3^+$	-	3.8	0.01	0
$CD19^+$	–	1.6	0.02	0
$CD33^+$	-	79.9	0.02	0

HCT, hematopoietic cell transplantation; mo, months; RTE, recent thymic emigrants; EM, effector memory; CM, central memory; EMRA, exhausted effector memory; PWM, pokeweed mitogen; ConA, concanavalin A; SM, switched memory; ↓, below normal values; ↑, above normal; #, inconclusive result where all T lymphocytes express intermediate levels of CD45RA and $CD31^+$.

lymphocyte response was not evoked to either tetanus or pneumococcus despite previous vaccinations, or candida despite positive cultures (Table 1). Assessment of the myeloid compartment (e.g., erythro-, mono-, and neutrophil granulocytes) was normal, besides absent eosinophil granulocytes and thrombocytosis (Table 1). Whole genome sequencing with an *in silico* filter for IEI (11) showed a heterozygous sequence variant in *IKZF1* (c.476A>G, p.Asn159Ser). Confirmatory Sanger sequencing of the patient and parents showed the mutation to be *de novo*.

Therapeutic intervention

At 10 months of age, our patient underwent allogeneic HCT with an unrelated 10/10 HLA-matched donor, the timeline is depicted in Figure 1. The PCJ pneumonia and respiratory viruses were cleared before admission. HCT conditioning consisted of fludarabine, treosulfan, and anti-thymocyte globulin. The HCT was performed with neutrophil and thrombocyte engraftment at days +36 and +24, respectively, without complications. Ciclosporin A and methotrexate were used as graft-versus-host disease (GVHD) prophylaxis. Six weeks after the procedure, cutaneous and gastrointestinal GVHD grade 1 occurred and was treated with prednisolone. Thereafter, CMV viremia occurred, with good response to pre-emptive therapy with valganciclovir. Because of mixed chimerism with low counts of T and B lymphocytes, an evaluating bone marrow biopsy was performed approximately +4 months post-HCT. It revealed near total $CD34^+$ autologous reconstitution, i.e., graft failure/rejection. Hence, re-HCT was performed with repeated conditioning (i.e., same as in first HCT with the addition of thiotepa) and the same donor and GVHD prophylaxis. Neutrophil and thrombocyte engraftment were seen at days +19 and +20, respectively. The re-HCT was without significant complications.

Follow-up and outcomes

Six months after re-HCT, the patient displayed full donor chimerism. An additional 12 months later, our patient experienced several uncomplicated upper airway infections, with spontaneous recovery. Twenty-seven months after re-HCT, the patient is alive and well, without any continuous treatment. She has a full numeric immune recovery (lymphocytes and immunoglobulins), responded adequately to vaccinations, is developing as expected, and is now in preschool.

Discussion

Our patient presented with evident IEI caused by a dominant negative mechanism providing loss of function in *IKZF1*, resulting in faltering lymphocyte development. This mechanism of action has previously been reported in presenting phenotypes similar to our patient's (12). The result is early-onset *IKZF1*-related IEI with a severe clinical phenotype (infections), characterized by somewhat functioning T lymphopoiesis (TREC generation and normal T lymphocyte counts), haltered T lymphocyte responses and memory generation, and a pronounced B lymphocyte defect. Although our patient's phenotype was presumably fatal if left untreated, and hence the CID could be regarded as severe, our patient did not meet the SCID criteria according to the Primary Immune Deficiency Treatment Consortium (PIDTC) 2022 Definitions for SCID (13). The criteria are based on absolute T

lymphocyte counts, not taking immune function and clinical phenotype into account. However, naming the disease SCID or pronounced early-onset CID might not impact the clinical care.

IKZF1-related CID has been described among at least seven patients previously reported by Boutboul et al. (10). Their phenotypes showed many similarities to our patient's, i.e., CID with lack of T lymphocyte activation and memory generation (e.g., abnormal T lymphocyte population with intermediate CD45RA expression and $CD31^+$) and low B lymphocyte levels, the evident major features of *IKZF1*-related CID. Boutboul et al. assessed TREC levels in three of their patients and they were normal to high (10), as in our patient, who had normal TREC levels. Our patient had a pronounced B lymphocyte development arrest, as seen in two of the patients in the Boutboul et al. cohort (10). The B lymphocyte arrest was reflected by the absence of KRECs, which has not been previously described in *IKZF1*-related CID. Additionally, we showed that our patient's B lymphocytes did not respond to PWM stimulation. Our patient contrasts to the previously reported ones with increased $CD4^+$ $V\beta2^+$ T lymphocytes, severely reduced $CD8^+$ T lymphocytes, reduced $CD8^+$ T lymphocytes proliferative response to mitogen (ConA), and no clear myeloid defects besides the unmeasurable eosinophils. However, regarding the latter, a slight myeloid defect might have been disguised by an inflammatory response to severe infection (i.e., PCJ pneumonia and respiratory tract virosis).

IKZF1-associated disease has previously been identified through TREC-based SCID NBS (9). However, our patient presented with normal levels of TREC and unmeasurable levels of KREC. The level of T lymphocytes was evidently enough to produce TRECs, and yet, presumably, this *IKZF1*-mutation provided a $CD4^+$ T lymphocyte dysfunction that led to an absence of both $CD8^+$ T and B lymphocytes. Additionally, there also seems to be an intrinsic B lymphocyte defect, as previously reported (14). In total, the combination of T and B lymphocyte dysfunction resulted in an absence of KRECs. The latter is not included in the Swedish national NBS program (4). It was, however, measured in this patient as a residual part of a research program (pilot) for SCID NBS and was hence available (6). If KREC analysis had been included in the NBS program, this patient would presumably have been detected at a much earlier stage. Early diagnosis is a strong contributing factor to a positive outcome for patients with severe early-onset IEI, wherefore including KREC determination in the NBS program would presumably improve the outcome for future patients with similar conditions. However, including KREC analysis in SCID NBS comes with a higher recall ratio and poor positive predictive values (6). This leads to a lot of stress for families of children with abnormal NBS samples that, after diagnostic workup, turn out to be normal or were reversibly abnormal due to maternal medication. Second tier testing with NGS, as is practiced in some countries in the case of low TRECs, might be an option to tackle these downsides (15, 16). In the case of *IKZF1*-related CID, lymphocyte phenotyping (i.e., abnormal T lymphocytes with intermediate CD45RA expression and $CD31^+$) as second tier or primary diagnostics could be a valuable tool in identifying these patients.

Our patient was successfully engrafted after re-HCT and displayed a full numeric immune recovery, with good response to vaccinations and lives a normal life. Hence, the profound CID seems to have been cured by the intervention. In mouse models of *IKZF1*-related CID, GVHD severity and HCT-related mortality were considerably high, so Kellner et al. compiled the first four known patients undergoing HCT due to *IKZF1*-related CID. These patients had a similar HCT outcome compared to our patient (i.e., upper normal to slightly delayed engraftment, treatable CMV viremia; 2/4, curable GVHD; 1/4, and full immunohematopoietic recovery in all surviving; 3/4) (17). Our patient rejected her first HCT graft after intermediate intensive conditioning. A rejection (1/4) without conditioning was also seen in the Kellner et al. cohort. These two rejecting patients may indicate that more intensive conditioning is needed in *IKZF1*-related CID, as used in our re-HCT and other reported engrafted patients (17).

As discussed, there are advantages and disadvantages to KREC determination in SCID NBS; however, this case illustrates the positive impact an inclusion of KRECs would have for patients with life-threatening early-onset CID.

Patient perspective

Today, our daughter looks and acts just as any three-year-old child on the outside. But it has not always been like this. Her life began brutal, with incredibly tough challenges and suffering. First and foremost, for her, but also for us as first-time parents, including everyone in our circle of acquaintances. There are no words that can describe how it feels as a parent when you think you are about to lose your child. If she could have been diagnosed earlier through NBS, we could have prevented severe and life-threatening infections, months of hospitalization, and shortened the already long time to recovery and eventually to the declaration of health. With that being said, our wish is that no other child and no family ever have to go through what we went through. Therefore, it is our sincere hope that all abnormalities that are linked to treatable serious diseases should be included in the NBS.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/clinvar/; RCV003326142.1.

Ethics statement

The studies involving humans were approved by Swedish Ethical Review Authority. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/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

CÄ: Investigation and Writing – original draft. RZ: Resources and Writing – review & editing. KR: Writing – review & editing. EA: Writing – review & editing. PM: Resources and Writing – review & editing. MS: Conceptualization, Funding acquisition, Supervision and Writing – review & editing.

Funding

The authors declare that financial support was received for the research of this article from the Samariten Foundation for Pediatric Research (Grant# 2022-21197).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 01 February 2024
DOI 10.3389/fimmu.2024.1329610

OPEN ACCESS

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RECEIVED 29 October 2023
ACCEPTED 15 January 2024
PUBLISHED 01 February 2024

CITATION
Al-Saud B, Alajlan H, Alruwaili H, Almoaibed L, Al-Mazrou A, Ghebeh H, Al-Alwan M and Alazami AM (2024) A unique STK4 mutation truncating only the C-terminal SARAH domain results in a mild clinical phenotype despite severe T cell lymphopenia: Case report.
Front. Immunol. 15:1329610.
doi: 10.3389/fimmu.2024.1329610

A unique STK4 mutation truncating only the C-terminal SARAH domain results in a mild clinical phenotype despite severe T cell lymphopenia: Case report

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Mutations in STK4 (MST1) are implicated in a form of autosomal recessive combined immunodeficiency, resulting in recurrent infections (especially Epstein-Barr virus viremia), autoimmunity, and cardiac malformations. Here we report a patient with an atypically mild presentation of this disease, initially presenting with severe T cell lymphopenia (< 500 per mm^3) and intermittent neutropenia, but now surviving well on immunoglobulins and prophylactic antibacterial treatment. She harbors a unique STK4 mutation that lies further downstream than all others reported to date. Unlike other published cases, her mRNA transcript is not vulnerable to nonsense mediated decay (NMD) and yields a truncated protein that is expected to lose only the C-terminal SARAH domain. This domain is critical for autodimerization and autophosphorylation. While exhibiting significant differences from controls, this patient's T cell proliferation defects and susceptibility to apoptosis are not as severe as reported elsewhere. Expression of PD-1 is in line with healthy controls. Similarly, the dysregulation seen in immunophenotyping is not as pronounced as in other published cases. The nature of this mutation, enabling its evasion from NMD, provides a rare glimpse into the clinical and cellular features associated with the absence of a "null" phenotype of this protein.

KEYWORDS

primary immunodeficiency, lymphopenia, NGS, SARAH domain, case report

Introduction

First identified in *Drosophila*, the Hippo pathway contains genes with close orthologues in mammals which are critical for organ development and growth (1). Serine-threonine kinase 4 (STK4), also known as mammalian sterile 20-like 1 (MST1), is the orthologue of *Drosophila* Hpo and is an essential kinase within the canonical and non-canonical Hippo signaling pathways. It promotes an assortment of immune cell functions including B cell immunity (2) and T cell expansion and migration (3, 4). Its downstream induction of FOXO (forkhead box protein) family members such as FOXO1 and FOXO3 allows STK4 to orchestrate effective CD8 T cell responses to persistent viral infections, the formation of regulatory T cells, and overall T cell homeostasis (5).

Bi-allelic loss-of-function mutations in STK4 cause combined immunodeficiency (CID) (6, 7). Deficiency of this protein has been linked to recurrent infections of bacteria, fungi, and viruses, with nearly half of the patients exhibiting Epstein-Barr Virus (EBV) viremia and EBV-associated lymphoproliferative disorder (8), exacerbated by the significantly impaired response of interferon types I, II, and III (9). Malignancies are often reported due to the subsequent development of B cell lymphomas. Sporadic neutropenia, T and B cell lymphopenia, and an elevated risk of autoimmunity are common findings (6, 7). Additional documented features include short stature, primary cardiac T cell lymphoma, and a Castleman-like disorder (9–11). Immunological characterization studies show that with time, memory B cell and naïve (CD45RA+) T cell numbers drop drastically, and peripheral T cell survival is severely compromised along with impaired response to antigens (7).

In addition to its kinase activity, STK4 protein contains a Salvador/Rassf/Hippo (SARAH) domain at its C-terminal end which is required for dimerization (12). Here we report a patient with profound T cell lymphopenia but otherwise mild clinical presentation. Her novel truncating mutation in STK4, situated further downstream than any previously described, generates an RNA transcript that is resistant to NMD and therefore illustrates the human phenotype that is associated with the loss of only the SARAH domain on the STK4 protein.

Case description

The patient is a 10-year-old female born to consanguineous parents from Saudi Arabia. The parents are asymptomatic and have a healthy 5-year-old son. The mother has a history of two first-trimester abortions for unknown reasons. At 4 years of age, the patient began having recurrent infections (accounting for three otitis media per year requiring antibiotics), plus urinary tract infections and recurrent oral thrush. Subsequently, she was admitted at the age of 7 years with severe gastroenteritis and her laboratory work up revealed leukopenia and normal immunoglobulin. She also experienced recurrent episodes of scalp hair loss and oral ulcers suspected to be secondary to nutritional deficit, which was managed by a course of omega-3 and zinc for 4 months with mild improvement. She also reported symptoms of recurrent frontal headache with photophobia and phonophobia (imaging was not done). The headache was relieved by rest and paracetamol, and was attributed to a family history of migraines. The patient had erythematous skin rash mainly on the trunk, sparing the face, and responded well to topical steroids.

At the age of 8 years, the patient was referred to us to investigate the possibility of primary immunodeficiency. At the time, physical assessment revealed weight and height within the 25th percentile. Chest examination revealed good bilateral air entry and no added sound, heart sound was normal and the remainder of the exam was unremarkable. Her initial laboratory results (Table 1) showed neutropenia, lymphopenia, and high IgM. Her T cell counts were severely depressed, being in the atypical or leaky SCID range. B cell count was also low, but with normal NK and acceptable response to tetanus vaccines.

She was evaluated by rheumatology for her history of recurrent hair loss, skin rash, and oral ulcers to rule out autoimmune diseases. Her initial autoimmune work up indicated normal ESR, negative for checked antibodies (anti-nuclear, anti-aPS aPT IgG/IgM, anti-endomyseal antibodies), normal anti tissue transglutaminase, anti-gliadin AB < 0.4, anti-cardiolipin IgA and IgG < 09.4, and normal C3 and C4 levels. These findings were not suggestive of any autoimmune disease. Echocardiography showed no cardiac anomalies.

She received all her vaccinations up to school age. There was no history of delayed milestones and she currently attends regular schooling.

The patient responded well to prophylactic antibiotic, antifungal, and intravenous immunoglobulin (IVIG) (0.4g/kg) every 4 weeks. She only had three admissions over the last 3 years with gastroenteritis; two were due to Clostridium difficile toxin, as stool samples were positive for toxigenic C. difficile by rapid DNA testing. The stool culture and stool for ova and parasites were otherwise negative, and the patient responded well to antibiotics.

TABLE 1 Initial immunological and hematological laboratory results.

Variable	Patient [a]	Normal range
WBC X 10^9/L	1.7	4.3 – 11.3
HB g/L	124	110 – 150
MCV fL	81.4	75 - 95
Platelet X 10^9/L	320	155 - 435
Neutrophils X 10^9/L	2.8	1.35 – 7.5
Lymphocytes X 10^9/L	1.04	1.9 – 4.9
Eosinophils X 10^9/L	0.18	0.03 – 1
IgG g/L	10.9	5.4 – 13.6
IgA g/L	2.31	0.5 – 3.05
IgM g/L	6.42	0.31 – 2.08
IgE KU/L	34.5	5 - 500
CD 3/mm^3	480	1700 - 1900
CD 4/mm^3	389	800 – 1700
CD 8/mm^3	72	700 - 1000
CD 19/mm^3	148	400 - 800
CD16/56/mm^3	161	200 - 400

[a]Sampled at the age of 8 years old.

Results

Identification of a novel STK4 mutation

We recruited this family under an IRB-approved informed consent (see Supplementary Materials). Our study encompassed the patient and her parents, but her unaffected sibling was not available for recruitment. Genomic DNA from the patient was analyzed through whole exome sequencing, then filtered under the assumption of an autosomal recessive disorder due to the high consanguinity rates in the region. Additional parameters were employed as shown in Figure 1A. A total of eight variants were then highlighted for segregation testing. Some variants could not be sequenced due to the repetitive nature of the locus, but given the lack of heterozygosity in the parents they were dismissed. Only two variants clearly segregated with the disease state, but one of these (*CEP295*:NM_033395.2:p.E2318K) was in a gene linked to primary microcephaly and Bardet-Biedl syndrome, and hence incompatible with our patient's presentation. The most promising variant was therefore a truncating mutation in the *STK4* gene (NM_006282.5: c.1311delG:p.S438*fs*), which was confirmed to be homozygous in the patient and heterozygous in her parents (Figure 1C).

Intriguingly, this frameshift mutation occurs in the last exon of *STK4*, which is more downstream than any of the other mutations reported for this gene to date (Figure 1B). The mutation is predicted to cause alteration and/or loss of the last 50 amino acid residues, which for a protein of this size (487 aa) constitutes just over 10%, and is expected to remove only the C-terminal SARAH domain, which participates in STK4 homo- as well as heterodimerization with other proteins (13). RT-PCR data revealed that transcripts of this gene were present in patient cells at nearly twice the quantity of our controls (Figure 1D) (details in Supplementary Materials). This significant difference suggested that the location of this variant in the last exon, just downstream of the final intron-exon boundary, was allowing the mutant transcript to escape NMD surveillance.

Immunoblotting of lysates from lymphoblastoid cells revealed the presence of the truncated form of STK4 in the patient. Contrary to the increased mRNA expression of this gene, the level of STK4 protein in the patient was significantly reduced (Figure 1E). FoxO3a, a direct downstream target of STK4 whose stability is dependent on STK4 phosphorylation, also showed low expression levels, and was at the lower range of what was observed in controls.

Flow cytometry analysis of patient T cells

Because of the role STK4 plays in modulating T cell health and activity, we subjected patient PBMCs to CFSE, a reagent that allows fluorescent staining of cells to track their proliferation index. T cells were then activated via the addition of CD3/CD28 Dynabeads and left to expand for 3 days (details in Supplementary Materials). Although the patient CD4+ cells did show proliferation, as evidenced by the decrease in overall CFSE signal intensity, it was significantly less than a panel of four healthy controls (Figure 2A). We note however that the dilution of CFSE over time is more noticeable in our patient cells than what has been described elsewhere (7), indicating a more robust response.

Additionally, as the literature indicates that STK4-deficient T cells show an exhausted phenotype prematurely (14), we also

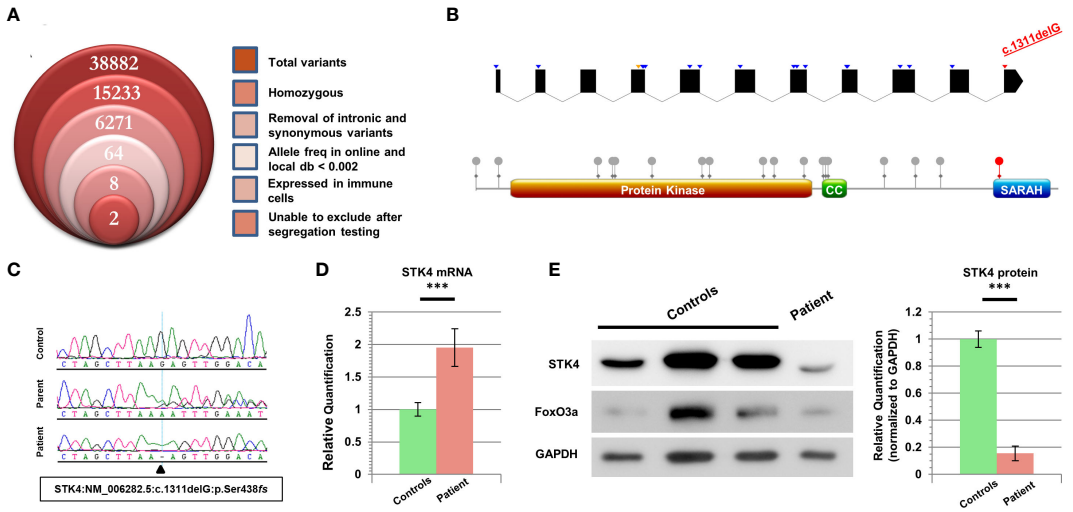

FIGURE 1
Characterization of cells harboring a novel *STK4* mutation. **(A)** Stacked Venn diagram indicating the total number of variants highlighted by WES, following the inclusion of each filter. **(B)** Top: schematic of the *STK4* gene illustrating the positions of previously reported mutations. Blue arrowheads denote truncating mutations, orange denotes missense, and our mutation is indicated in red. For the sake of clarity, UTR regions are not shown, and intronic regions are not drawn to scale (although coding portions of exons are to scale). The list of mutations is based on HGMD Professional (v 2023.1). Bottom: schematic of the STK4 protein highlighting the locations of domains and reported coding mutations. Our mutation is the only one whose impact is limited to loss of the C-terminal SARAH domain. **(C)** Sequence chromatograms of the patient, one parent, and a healthy control for comparison. **(D)** Real-time RT-PCR data for STK4 expression levels in the patient's lymphoblastoid cells, versus combined data from three healthy controls. **(E)** Left: immunoblotting reveals low levels of truncated STK4 protein in the patient's lymphoblastoid cells, as well as low levels of the downstream target FoxO3a. GAPDH serves as a loading control. Right: quantification of three independent immunoblots, utilizing ImageJ analysis, reveals that STK4 protein expression is significantly depressed in the patient. ***$p < 0.001$; unpaired Student's *t*-test.

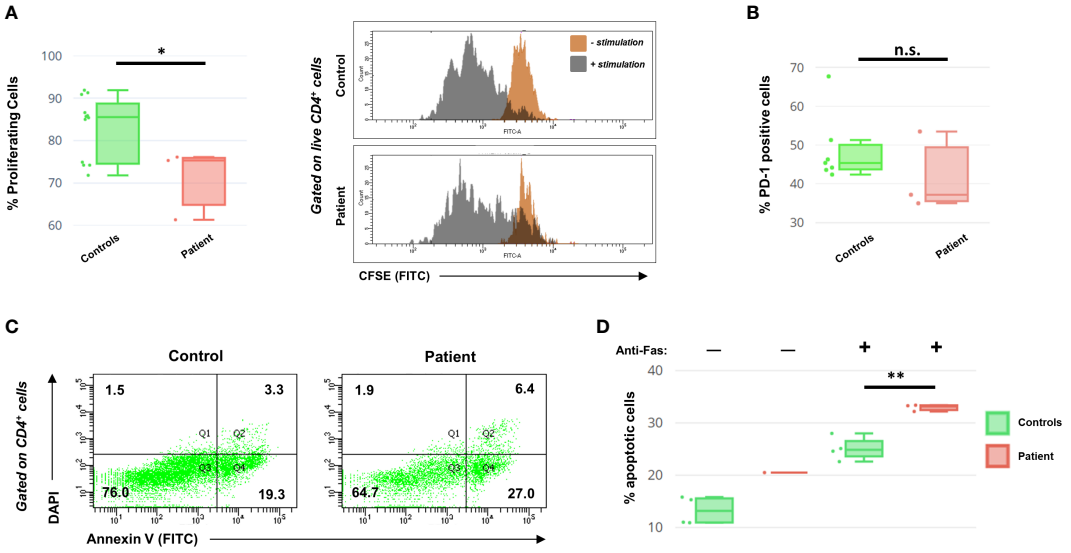

FIGURE 2
(A) Left: Box-and-whiskers summary of CFSE proliferation data on PBMCs from controls and the patient following 3 days of CD3/CD28 stimulation and gated on live (DAPI-) CD4+ T helper cells. Right: representative histograms of CFSE readout for CD4+ cells, in the presence or absence of stimulation. **(B)** PD-1 cell surface expression data, following 48 hours of CD3/CD28 stimulation. Data are presented as a percentage of live CD4+ cells. **(C)** Representative histograms of the DAPI/Annexin V cellular profile for patient and control CD4+ cells, following T cell activation with anti-Fas antibody challenge. The quadrants relate to necrosis (Q1), late apoptosis (Q2), healthy (Q3), and early apoptosis (Q4). Numbers indicate cell percentages within each quadrant. **(D)** Summary of the AICD data for activated control and patient CD4+ cells, following 72 hours of CD3/CD28 stimulation with or without 24 hours of anti-Fas incubation. Percentage shows the sum of both early and late apoptotic cells. Annexin V profiling of patient T cells in the absence of anti-Fas was conducted only once due to cell number limitations. Asterisks indicate significance levels (*$p < 0.05$, **$p < 0.01$; unpaired Student's *t*-test). Error bars for **(A, B, D)** indicate SEMs. n.s., not significant.

investigated this in our patient. T cells were stimulated for 2 days before assessing the helper T cell fraction for expression of PD-1, a surface marker for cellular exhaustion. Patient CD4+ cells did not show evidence of exhaustion (Figure 2B). This suggests that the increased PD-1 observed in other published STK4 patients may be a consequence of persistent EBV viremia, more than being an intrinsic part of the disease.

Loss of STK4 in both mice and humans is known to negatively impact T cell survival through increased apoptosis (6, 7, 15). We therefore evaluated activation-induced cell death (AICD) in the patient's T cells by examining the effect of Fas binding on activated T lymphoblasts. PBMCs were activated for 48 hours with CD3/CD28 Dynabeads and then were mixed with or without anti-Fas antibody for an additional 24 hours before analysis of the DAPI/Annexin V apoptotic profile. In the absence of anti-Fas, the patient's cells exhibited a greater propensity for apoptosis than controls (conducted only once due to cell limitations). In the presence of anti-Fas, both the control and the patient's $CD3^+$ cells displayed increased apoptosis, with the patient's cells being significantly more susceptible to AICD (Figures 2C, D).

Immunophenotyping reveals widespread dysregulation

To functionally assess the immunological effects of this mutation on our patient, PBMCs from the patient and age-matched healthy controls were stained with a panel of fluorescent antibodies prior to assessment using flow cytometry. This enabled us to evaluate the major lymphocyte populations, as well as specific T and B cell compartments (Figure 3). FACS analysis revealed widespread dysregulation of various populations, including strong decreases in the percentages of $CD3^+$ T cells, $CD8^+$ cytotoxic T cells, and naïve T cell compartments subtypes. Conversely, the effector memory subtypes of both $CD4^+$ and $CD8^+$ T cells were drastically overrepresented. Amongst B cells there was a trend of a high percentage of naïve cells and a low percentage of memory cells. The plasmablast subpopulation was substantially amplified as reported in other patients (16), although, unlike the overwhelming majority of cases, our patient exhibited normal IgG but high IgM levels. No obvious changes were detected in the NK or T_{reg} percentages as compared to controls.

Discussion

To the best of our knowledge, this is the first report to showcase functional work on STK4-deficient human cells that still contain residual STK4 protein (i.e. are not a null phenotype). Previous mutations largely involved truncating or splice site changes that led to mRNA degradation and loss of detectable protein (6, 7, 11, 14, 17).

Our patient's mutation is unique in that it removes the C-terminal SARAH domain only, and is present in the last exon which makes it resistant to NMD surveillance. In fact, while the amount of STK4 mRNA that is generated is significantly higher than in

FIGURE 3
Immunophenotyping of the patient's PBMCs versus a cohort of healthy pediatric controls (n = 6) using flow cytometry. Box-and-whiskers indicate major lymphocyte populations as well as $CD4^+$ and $CD8^+$ T cell, and $CD19^+$ B cell compartments. Subpopulations for which our patient's percentage is at variance with the combined data of the controls are boxed in red. Variance in data between this figure and Table 1 (sampled roughly 1 year apart) is likely due to different sampling dates and changes in therapy, as well as the use of different protocols, antibody clones, fluorescent color combinations, and flow cytometers inherent to the clinical service versus the research laboratory.

controls, the level of detected protein is significantly lower. The SARAH domain functions as a hydrophobic platform upon which proteins are able to form both hetero- and homodimers in anti-parallel orientation (13). Homodimerization, in particular, is crucial for allowing STK4 proteins to autophosphorylate each other, leading to their nuclear translocation and subsequent induction of cellular apoptosis via DNA fragmentation. The overwhelming majority of naturally occurring STK4 is found to be homodimerized, indicating that the monomeric form is not favored (18). We theorize that in our patient's cells, loss of the SARAH domain and the consequent inability to undergo efficient autophosphorylation may have led to decreased stability and hence the reduced protein levels we observed.

STK4 activity can be modulated through the phosphorylation of certain residues contained within the SARAH domain (13). Interestingly, our patient's mutation directly abolishes one of these, Ser438, which is an mTORC2 target that serves to inhibit STK4 and thereby facilitate cell proliferation. Phosphorylation of the nearby Thr440 triggers the opposite function by increasing STK4 activity (19, 20). It is intriguing to wonder if (and how) protein behavior might have changed had the frameshift mutation been located slightly further downstream, leaving one or both of these residues intact.

At both the clinical and cellular levels, there are remarkable differences between our patient and the consensus outlook reported in the literature. For example, STK4 deficiency is often associated with reduced IgM while our patient exhibits hyper IgM, a finding that has only ever been documented in one other case (17). Although our patient's T cells are deficient in proliferation against a cohort of controls, following stimulation, they show similar speed to some slower controls (Figure 2A), unlike other patients in which the divergence is much more pronounced (7). Likewise, our patient's T cells showed increased levels of apoptosis following continued stimulation (especially when challenged with anti-Fas, Figure 2D), but the percentage of apoptotic cells is not as high as cited elsewhere (6, 7).

It is interesting to note the duality of roles taken up by STK4 in immune cells. Aside from its function as tumor suppressor, it plays an anti-apoptotic role in hematopoietic cell lineages, protecting T cells from undergoing excessive apoptosis in knockout mice (15). Similarly, in humans, the null condition leads to dysregulation of immunophenotype and suppressed lymphocyte counts. Although this was noted in our patient, the extent was less severe than other reports. For example, the central memory compartments of both $CD4^+$ and $CD8^+$ T cell subpopulations, are not outside the bounds of what we observed in a healthy pediatric population, unlike the consensus of STK4 patients (16).

Patients with STK4 deficiency exhibit variable laboratory and clinical characteristics, and in accordance with previously published cases our patient demonstrated some of these common features such as intermittent neutropenia, and recurrent sinopulmonary infections. The latter, however, was controlled with immunoglobulin replacement therapy and prophylactic antibiotics. CD4 lymphopenia, the single most consistent finding in the literature, was indeed detected (Table 1) but not always consistently (Figure 3). Moreover, she was negative for other established features such as autoimmune disease, repeated skin viral infections, atopy, and EBV-associated lymphoproliferative disease. We hypothesize that this milder clinical phenotype, going hand-in-hand with the milder cellular phenotype, reflects the lack of NMD coupled with the presence of a low basal level of phosphorylation (in the absence of a SARAH domain that would facilitate full autophosphorylation). This leads to a partially rescued phenotype as compared to "null" cases.

Some of the previously reported patients exhibited congenital heart defects, however our patient's echocardiography revealed no cardiac anomalies. In a recent cohort, all patients were evaluated by echocardiography regardless of symptoms and none were found to have heart defects. The authors concluded that the previously noted presence of cardiac abnormalities in STK4 patients may be coincidental (16).

A review of the literature reveals that 10 out of 26 reported patients underwent allogeneic hematopoietic stem cell transplantation (HSCT) (16, 21), with a post-transplant survival rate of 50%. Of these 10 patients, at least 7 were referred for transplant primarily due to EBV viremia/lymphoma. Our patient had no full-matched related stem cell donor, and a further search for unrelated stem cell sources was not done given the milder disease course.

Our data indicate that the presence of the STK4 peptide, in a truncated form that is missing the C-terminal SARAH domain, leads to largely improved clinical and laboratory findings as compared to the usually-reported "null" phenotype. Further work will need to be done to determine whether some patients, after considering the nature and location of their mutation, might benefit from future therapies that selectively modulate NMD activity. Such therapies may allow for the expression of truncated, partially-functional STK4 peptides with a stabilizing effect on lymphocyte survival and overall health.

Data availability statement

The datasets presented here are protected under local data privacy laws, and cannot be made publicly available. Requests to access the datasets should be directed to the corresponding author (amalazami@kfshrc.edu.sa).

Ethics statement

The King Faisal Specialist Hospital & Research Centre (KFSHRC) institutional review board approved all patient-related research, RAC # 2080 025. Informed consent was acquired from the parents for themselves as well as for the patient (as her legal guardians). Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

BA: Data curation, Supervision, Writing – review & editing. HuA: Data curation, Investigation, Methodology, Writing – review & editing. HiA: Data curation, Investigation, Methodology, Writing – review & editing. LA: Data curation, Writing – review & editing. AA: Data curation, Writing – review & editing. HG: Data curation, Investigation, Writing – review & editing. MA: Data curation, Formal analysis, Investigation, Supervision, Writing – review & editing. AMA: Formal analysis, Methodology, Project administration, Supervision, Writing – original draft.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We thank the family members for their generous participation. We are grateful to Dorota Monies and the sequencing core facility at KFSH&RC for their invaluable assistance, to Sawsan AbuAwwad for help with the recruitment process, and to Asma Tahir for insightful discussions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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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.2024.1329610/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 28 June 2024
DOI 10.3389/fimmu.2024.1428724

OPEN ACCESS

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RECEIVED 06 May 2024
ACCEPTED 17 June 2024
PUBLISHED 28 June 2024

CITATION
Boyarchuk O, Kostyuchenko L, Akopyan H, Bondarenko A, Volokha A, Hilfanova A, Savchak I, Nazarenko L, Yarema N, Urbas O, Hrabovska I, Lysytsia O, Budzyn A, Tykholaz O, Ivanchuk M, Bastanohova O, Patskun E, Vasylenko N, Stepanovskyy Y, Chernyshova L and Makukh H (2024) Nijmegen breakage syndrome: 25-year experience of diagnosis and treatment in Ukraine. *Front. Immunol.* 15:1428724. doi: 10.3389/fimmu.2024.1428724

Nijmegen breakage syndrome: 25-year experience of diagnosis and treatment in Ukraine

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Introduction: Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder, characterized by microcephaly, immunodeficiency, and impaired DNA repair. NBS is most prevalent among Slavic populations, including Ukraine. Our study aimed to comprehensively assess the prevalence, diagnosis, clinical data, immunological parameters, and treatment of NBS patients in Ukraine.

Methods: We conducted a retrospective review that included 84 NBS patients from different regions of Ukraine who were diagnosed in 1999-2023. Data from the Ukrainian Registry of NBS and information from treating physicians, obtained using a developed questionnaire, were utilized for analysis.

Results: Among 84 NBS patients, 55 (65.5%) were alive, 25 (29.8%) deceased, and 4 were lost to follow-up. The median age of patients was 11 years, ranging from 1 to 34 years. Most patients originate from western regions of Ukraine (57.8%), although in recent years, there has been an increase in diagnoses from central and southeastern regions, expanding our knowledge of NBS prevalence. The number of diagnosed patients per year averaged 3.4 and increased from 2.7 to 4.8 in recent years. The median age of NBS diagnosis was 4.0 years (range 0.1-16) in 1999-2007 and decreased to 2.7 in the past 6 years. Delayed physical

development was observed in the majority of children up to the age of ten years. All children experienced infections, and 41.3% of them had recurrent infections. Severe infections were the cause of death in 12%. The second most common clinical manifestation of NBS was malignancies (37.5%), with the prevalence of lymphomas (63.3%). Malignancies have been the most common cause of death in NBS patients (72% of cases). Decreased levels of CD4+ and CD19+ were observed in 89.6%, followed by a reduction of CD3+ (81.8%) and CD8+ (62.5%). The level of NK cells was elevated at 62.5%. IgG concentration was decreased in 72.9%, and IgA - in 56.3%. Immunoglobulin replacement therapy was administered to 58.7% of patients. Regular immunoglobulin replacement therapy has helped reduce the frequency and severity of severe respiratory tract infections.

Conclusion: Improvements in diagnosis, including prenatal screening, newborn screening, monitoring, and expanding treatment options, will lead to better outcomes for NBS patients.

KEYWORDS

Nijmegen breakage syndrome, *NBN* gene, c.657_661del5 variant, malignancies, diagnosis, incidence, clinical analysis, immunological characterization

Introduction

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder classified among inborn errors of immunity (IEI), characterized by microcephaly, immunodeficiency, impaired DNA repair, leading to high sensitivity to ionizing radiation and an increased risk of malignancy.

Chromosomal instability syndrome was first highlighted in the article by Hustinx et al. (1), where a patient with microcephaly, growth delay, selective IgA deficiency, and chromosomal rearrangements was reported. The etymology of the disorder's name is associated with the city of Nijmegen, where the first two cases of NBS were described in 1981 (2).

NBS is most prevalent in Eastern European populations, particularly in Poland, the Czech Republic, and Ukraine, where the frequency of homozygous carriage of the "Slavic" pathogenic variant (c.657_661del5) of *NBN* gene approaches 1:155 (3). The prevalence of NBS in Ukraine is 1.3 per 1,000,000 population, with the highest frequency of cases in western regions (up to 20 cases per 1,000,000 individuals) (4). NBS is also common in isolated Slavic groups in southeastern Germany, where the carrier frequency is 1:34 (5). In the United States, carriers of the pathogenic variant c.657_661del5 are usually patients of Eastern European descent (6).

Malignancy is a manifestation of NBS and is sometimes the presenting symptom in heterozygous carriers of the pathogenic variant of the *NBN* gene (2). *NBN* gene in NBS encodes the protein Nibrin, which is part of the NBN/Mre11/Rad50 complex and, interacting with ATM kinase, halts the cell cycle with DNA repair. Double-strand DNA breaks may occur during normal replication processes and as a result of the impact of damaging agents on DNA (7).

In patients with NBS, inefficient repair of DNA breaks occurs in the absence of Nibrin, disrupting V(D)J recombination processes during lymphocyte differentiation (8). Impaired lymphocyte proliferation and differentiation lead to a decrease in their number, the formation of antigen-recognition domains of immunoglobulins, and the T-cell receptor (9).

NBN plays a crucial role in neurogenesis (9, 10). DNA damage affects the development of neurons in various parts of the brain. Microcephaly can be caused by alterations in neural progenitor cells in response to DNA damage. Impaired cell proliferation and differentiation, and the absence of Nibrin in NBS patients result in growth retardation (11).

Chromosomal instability has been demonstrated in the karyotype of patients with NBS in several clinical studies (1, 12–14), most often revealing inversions and translocations involving two different loci on chromosomes 7 and 14 (14, 15). Breakpoints are located in the chromosomal bands 7p13, 7q35, 14q11, and 14q32, regions of T-cell receptor genes and the human immunoglobulin heavy chain gene. Cytogenetic studies in NBS patients' lymphocytes reveal fusion of the ends of adjacent chromosomes, indicating telomere dysfunction (16, 17). Since telomere shortening triggers cell aging, the role of Nibrin in telomere maintenance likely also contributes to growth retardation in NBS patients.

Thus, studies have shown the widespread prevalence of NBS among Slavic populations and identified the role of the Nibrin protein in the clinical manifestations of the disease. The wide

prevalence of NBS poses important tasks for doctors and scientists in Ukraine: establishing molecular-genetic diagnostics of the syndrome, adequate clinical management of patients to prevent oncological and other complications of congenital combined immunodeficiency, and increasing attention to NBS among children with microcephaly.

Previous studies examined the course of NBS in a cohort of 26 patients from 1999-2007 (18). In addition, 14 patients from the Lviv region included in the ESID registry were included in the study by Wolska-Kuśnierz et al. in 2015 (19), and 56 patients from Ukraine were included in the cohort of Eastern Slavic population NBS patients, which studied the prevalence of malignancies (4). Hence, there have been no comprehensive investigations conducted in recent years on the prevalence, clinical presentations, and complications of NBS within the Ukrainian population. Our study aimed to comprehensively assess the prevalence, diagnosis, clinical data, immunological parameters, and treatment of NBS patients with 657del5 variant in *NBN* gene in Ukraine.

Materials and methods

Patients

We conducted a retrospective study that included 84 NBS patients from different regions of Ukraine. In 82 patients, the diagnosis was suspected based on clinical data, and in two patients - based on positive neonatal screening results. The diagnosis was confirmed by genetic testing in all patients. In 75 cases, the diagnosis was confirmed at the Institute of Hereditary Pathology in Lviv, Ukraine; in four cases - at the Scientific Medical Genetic Center LeoGENE, Ukraine; in three cases - at the Invite laboratory, USA; in one case - at the genetic laboratory of the National Specialized Children's Hospital "OHMATDYT", Kyiv; and in one case - at a clinic in Italy, where hematopoietic stem cell transplantation (HSCT) was performed. A homozygous c.657_661del5 variant in *NBN* gene was detected in 83 patients, and heterozygous c.657_661del5 and c.995-2A>G variants were found in one patient.

The research adhered to the guidelines outlined in the 1975 Declaration of Helsinki (revised in 2000) and received approval from the Ethics Committee of I. Horbachevsky Ternopil National Medical University (Minutes № 75). All participants and/or their parents provided written informed consent.

To analyze demographic data and clinical signs, we used data from the Ukrainian Registry of IEI (20). We also surveyed immunologists with experience observing patients with NBS, using a detailed questionnaire developed by Professor Larysa Kostyuchenko. This questionnaire included questions about various aspects of the disease: clinical features, laboratory indicators, and treatment methods.

Evaluating previous studies on NBS in Ukraine, we divided our analysis into three periods: 1999-2007, 2008-2017, and 2018-2023. This approach allowed us to reconcile data and identify certain patterns in the disease dynamics.

Additionally, to prevent new cases of NBS in high-risk families, a prenatal diagnostics program has been developed at the Institute of Hereditary Pathology. The program has already been successfully implemented in five families of heterozygous carriers of c.657_661del5 variant in the NBN gene.

Genetic diagnosis

Molecular-genetic analysis of the pathogenic variant c.657_661del5 of the *NBN* gene was conducted in probands with clinical suspicion and phenotypic features of NBS. DNA isolation and purification were performed using the phenol extraction method and salting-out method. For postnatal diagnosis, venous blood samples collected in EDTA tubes or dried blood spots (DBS) were used. In cases of prenatal diagnosis, DNA was isolated from chorionic villi or amniotic fluid. *In vitro* DNA sequence amplification was performed using the polymerase chain reaction (PCR) method with the following primer sequences 5'-CAGATAGTCACTCCGTTTACAA-3' and 5'-TTACCTGTTTGG CATTCAAA-3' and DreamTaq PCR MM (Thermo Scientific). DNA fragment separation was carried out by DNA electrophoresis in a 10% polyacrylamide gel prepared in Tris-borate buffer. Electropherograms were scanned on an ultraviolet transilluminator. The obtained signals were compared with length markers, and based on this, the sizes of the obtained fragments were detected. In the presence of the pathogenic variant c.657_661del5 of the *NBN* gene in a homozygous state, the size of the PCR fragment was 176 bp, compared to the normal allele - 181 bp. Sanger Sequencing was performed using the QuantumDye sequencing kit (Quantum Seq) on SeqStudio Capillary electrophoresis (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using STATISTICA 10. We used descriptive statistics. Qualitative variables are shown as absolute frequencies and percentages. Quantitative variables were tested using the Kolmogorov–Smirnov test or histogram for normal distribution and are expressed as median and interquartile range (IQR), when appropriate. For categorical data, chi-squared tests were used. Values of $p<0.05$ were considered significant.

Results

Characteristics of NBS patients

Among 84 NBS patients, there were four pairs of siblings. Forty-four were males (52.4%). The median age of all patients was 11 years, ranging from 1 to 34 years. Overall, 61.9% of patients were alive at the time of data collection, with 28 (33.3%) deceased. Four patients were lost to follow-up for various reasons. The median age of live patients was 12 years, ranging from 1 to 34 years. Twelve

living patients are currently over 18 years old (21.4%), and four patients are over 30 years old. The median age of deceased patients was 9 years, ranging from 4 to 16 years.

The distribution of patients according to the regions of Ukraine is shown in Figure 1.

Analysis of diagnosed patients depending on the region of Ukraine was conducted. Overall, the largest proportion of patients (57.8%) is registered in the western regions, but in recent years, there has been a trend of increasing the number of patients in the central and northern regions: from 0% in the period 1999-2007 to 35.7% in the last 6 years (Figure 2).

The number of diagnosed patients per year averaged 3.4 and increased from 2.7 to 4.8 in recent years (Table 1). The median age of NBS diagnosis from 1999 to 2023 was 4.0 years (range 0.1-16). It decreased from 4.0 years in 1999-2007 and 5.5 years in 2008-2017 to 2.7 years in 2018-2023. The median age of diagnosis also varied depending on the region: in western regions, it was 2.6 years (range 0.1-16 years), in central and northern regions – 5.5 years (range 0.1-13 years), and in southern and eastern regions – 6 years (range 0.4-16 years); p=0.0838 between western and other regions.

Prenatal diagnosis and medical-genetic counseling

Prenatal diagnosis were carried out in 5 families of heterozygous carriers of pathogenic variant c.657_661del5 of *NBN* gene. In the family where two children with NBS died from complications of Hodgkin's lymphoma, 4 prenatal diagnostic procedures were conducted. In 2 cases, a homozygous pathogenic variant in the *NBN* gene was identified, while in the other two cases, a heterozygous variant and normal *NBN* genotype were identified, and healthy children were born. In the remaining four families at high risk of NBS, prenatal diagnosis was performed only once, during the subsequent pregnancy after the birth of a child with NBS. A homozygous pathogenic variant of *NBN* was identified in 3 of the investigated cases, and only one healthy child was born in one family.

Clinical manifestations

Data on the clinical course of NBS were provided for 58 patients (69.0%). Microcephaly was noted in all, 4 (6.9%) children were born prematurely, but in 41 (70.7%) newborns, the birth weight was less than 3000 g, while the body length was less than 50 cm in 25 (43.1%) children. The median birth weight was 2800 g, ranging from 1800 to 3800 g (Table 2). The median body length at birth was 50 cm, ranging from 44 to 54 cm. At birth, only one patient had a head circumference of 34 cm, which corresponds to the lower limit of the norm, while in the rest, it ranged from 27 to 33 cm, with a median of 30 cm. Subsequently, microcephaly was observed in all children, with a median head circumference deviation of -6.5z at 1 year, ranging from -4.3 to -8.1z, and a median of -6.1z at 5 years, ranging from -3.8 to -7.5z.

Delayed physical development was observed in the majority of children up to the age of 10 (Table 3). Deviations in weight were more frequent and pronounced than in body length/height. After 10 years, a positive trend was observed in both weight and height.

Dysmorphic facial features, such as characteristic faces, sloping forehead, prominent and/or hooked nose, small chin, upslanting palpebral fissures, and abnormal ears, were observed with varying frequency (Table 3). The predominant skin manifestations included café au lait spots (46.6%) and vitiligo (24.1%).

All children experienced infections; however, in 34 (58.6%) cases, they were sporadic during the observation period, did not require intensive medication intervention, and manifested as common infections. In 24 (41.3%) cases, the infections were recurrent. Among them, respiratory tract infections were most common: bronchitis (27.6%) and sinusitis (17.2%). Pneumonia

FIGURE 1
Geographical distribution of NBS patients (n) by regions of Ukraine.

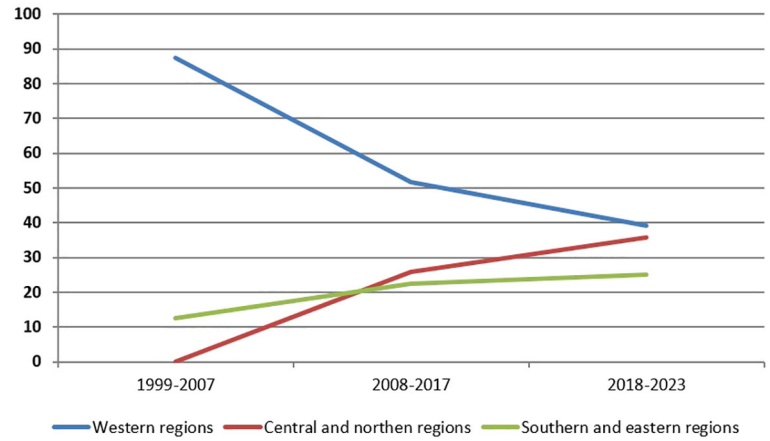

FIGURE 2
Trends in diagnosing NBS patients (%) depending on the period (years) and region.

was reported in 22.4% of children. Recurrent otitis media (22.4%), stomatitis (13.8%), and urinary tract infections (UTIs) (8.6%) were frequently reported by physicians. Herpes simplex infections, mainly labialis, were observed in 13.8% of children. Four children had hepatitis (hepatitis B and hepatitis C in two children each), two had sepsis unrelated to immunosuppression in HSCT, and one child had tuberculosis.

Information about COVID-19 was available for three patients. In children receiving immunoglobulin replacement therapy, the disease had a mild course. In an adult patient (34 years old), COVID-19 manifested with high fever and respiratory symptoms but did not require hospitalization. After SARS-CoV-2 infection, this patient developed arthritis of the knee joint.

Severe infections were the cause of death in three children (12%).

The second most common clinical manifestation of NBS was malignancies, which occurred in 35 out of 80 patients (43.8%) at the time of examination. Of the 35 patients with malignancies, 24 were boys (68.6%) and 11 were girls (31.4%), p=0.0399.

Among malignancies, lymphomas were most commonly observed (62.9%), followed by leukemias (22.9%), and in isolated cases, other types of malignancies were identified (rhabdomyosarcoma, ovarian cancer). In three cases, the diagnosis was unclear. Of the 22 patients with lymphomas, 18 had non-Hodgkin lymphomas (NHL) and 4 had Hodgkin lymphoma. Among the NHL, diffuse large B-cell lymphoma (DLBCL) was observed in 10 patients, other types of B-cell lymphoma – in 3, T-cell lymphoblastic lymphoma (T-LBL) – in 4, and peripheral T-cell lymphoma (PTCL) – in one patient. The median age of children at the time of malignancy diagnosis was 9.5 years, ranging from 3 to 15 years. Currently, 22 patients (62.9%) with malignancy have passed away, while the rest are in remission or continue treatment.

Autoimmune and allergic manifestations in NBS patients were less common, observed in 15.5% and 8.6% respectively (Table 4). Among autoimmune phenomena, arthritis (6.9%), neutropenia (6.9%), and psoriasis (1.7%) were noted. Allergies included atopic dermatitis (4; 6,9%), allergic rhinitis (2; 3.4%), and bronchial asthma in 1 child.

Immunological examination

Immunological data were provided for 48 (57.1%) NBS patients. Decreased lymphocyte levels were observed in 64.8% of them (Table 4). The most common reductions were in absolute values of CD4+ and CD19+ (89.6%), followed by CD3+ (81.8%) and CD8 + (62.5%). Meanwhile, the level of NK cells was more frequently elevated (62.5%). The most common reduction among immunoglobulins was in IgG concentration (72.9%), followed by

TABLE 1 Dynamics of the number of born and diagnosed NBS patients and median age of diagnosis.

Criterion	1999-2007	2008-2017	2018-2023	Total
Number of diagnosed patients, n	24	31	29	84
Number of diagnosed patients per year	2.7	3.1	4.8	3.4
Age at diagnosis, years; Me (range)	4.0 (0.1–16)	5.5 (0.1-16)	2.7 (0.1-13)	4.0 (0.1–16)
Number of born patients	28	31	12	71*
Number of newborns, thousands	3,737	4,686	1,618	10,041
Incidence in newborns	1: 133,000	1: 151,000	1: 135,000	1:141,000

*13 NBS patients were born in 1989-1998 years.

TABLE 2 Physical development of NBS patients depending on age.

Age, years	Me	IQR	Range (min; max)	Less than -2z	n
Weight, z-score					
At birth	2800 g	2580; 3050 g	1800; 3800 g		58
1	-1.97	-2.25; -0.77	-5.03; 2.0	19; 55.9%	34
3	-2.30	-4.56; -1.16	-8.03; 2.71`	13; 59.1%	22
5	-2.07	-3.73; 0.72	-7.87; 3.83	16; 64%	25
10	-1.93	-2.93; 0.18	-4.0; 2.28	10; 56.3%	16
14	0.19	-0.39; 1.18	-4.28; 1.18	3; 33.3%	9
18	0.29		-0.57; 0.1		2
Length/height, z-score					
At birth	50 cm	48; 51 cm	44; 54 cm		58
1	-1.17	-2.69; -0.11	-4.05; 0.77	13; 41.9%	31
3	-0.47	-1.6; 1.02	-5.88; 4.90	6; 28.6%	21
5	-0.71	-2.60; 0.67	-7.64; 0.89	8; 33.3	24
10	-1.80	-2.50; 1.17	-4.20; -0.84	7; 50%	14
14	-0.91	-1.44; 0.10	-2.18; 0.10	1; 12.5%	8
18	-0.31		-3.7; 0.31		2

IgA (56.3%). The median value of IgG was also low, at 3.7 g/l. Immunoglobulin M was within the normal range in most cases, but in 20.8% it was elevated, and only in 8.3% it was decreased. In 60% of children with measured IgE, it was decreased, but in 4/30 (13.3%) it was elevated, and in 2 patients it exceeded 1000 IU/ml.

Treatment

There is currently no specific treatment for NBS. The main methods of treatment for NBS patients include immunoglobulin replacement therapy, prophylactic antibiotic therapy, and HSCT. HSCT has shown clinical benefits for immunodeficiency and hematologic cancer in patients with NBS (21). However, the high risks associated with this treatment are linked to increased radiosensitivity and impaired tolerance to chemotherapy. There is also a risk of secondary malignancies (21). Additionally, ethical considerations must be taken into account, especially when neurological impairments progress, as congenital chromosomal fragility and radiosensitivity, will remain unchanged even with a successful HSCT. Therefore, the decision to pursue transplantation should be considered individually for each patient, taking into account the clinical severity of immune defects and other manifestations.

Immunoglobulin replacement therapy was administered to 37/63 (58.7%) NBS patients with low IgG levels and/or recurrent and severe infections. Patients received regular immunoglobulin at a 400-800 mg/kg dose once every 28 days. Regular prophylactic immunoglobulin replacement therapy became available in Ukraine only in 2006. Until then, immunoglobulin replacement therapy was episodic. The median age of starting immunoglobulin therapy was 3.5 years, ranging from 3 months to 16 years. Prophylactic antibiotic therapy was administered in 13/63 (20.6%) patients with recurrent respiratory symptoms. Other treatment options included antiviral and antifungal therapy when necessary.

HSCT was performed in 4 patients, three of whom died. HSCT was performed in Italy, Poland, Turkey, and Ukraine. In three patients, it was performed in remission of malignancies, while in one child, considering the complicated infectious history, progressive decline in T- and B-lymphocytes.

Unfortunately, the first experience of HSCT in Ukraine in a 3-year-old NBS patient was unsuccessful. The patient did not have malignancy but had a severe recurrent infection and progressive T-cell lymphopenia. A compatible related donor was not available. With the help of the Ukrainian bone marrow donor registry, a compatible non-relative donor (9/10 match at the A locus) was found in the International bone marrow donor registry, as a 10/10 match donor was unavailable. After comprehensive pre-transplant evaluation, the patient underwent reduced-intensity conditioning (RIC) according to the EBMT/ESID Guidelines for HSCT for primary immunodeficiency (modified preparative regimen for patients with radiosensitivity). Engraftment was achieved within the expected timeframes: neutrophil on day +11, platelet on day +13, erythrocyte on day +15. Molecular genetic study of donor chimerism in peripheral blood by PCR on days +27, +57, +90, and +197 confirmed complete donor chimerism - 100%.

Starting from the early post-transplant period, the patient experienced immune complications: graft-versus-host disease (GVHD), which was resistant to glucocorticoid therapy. Therapy with mesenchymal stem cells, vedolizumab also did not have a

10.3389/fimmu.2024.1428724

TABLE 3 Frequency of clinical manifestations in NBS patients (n=58).

Sign	n	%
Craniofacial abnormalities	**58**	**100**
microcephaly	58	100
characteristic faces	55	94.8
sloping forehead	56	96.6
prominent and/or hooked nose	39	67.2
small chin	54	93.1
upslanting palpebral fissures	30	51.7
abnormal ears	46	79.3
Skin manifestations		
café au lait spots	27	46.6
nevi	4	6.9
vitiligo	14	24.1
Infections		
Sporadic respiratory tract infections	34	58.6
Recurrent infections	24	41.3
bronchitis	16	27.6
otitis	13	22.4
sinusitis	10	17.2
pharyngitis/tonsillitis	4	6.9
chronic bronchitis/bronchoectasis	4	6.9
stomatitis	8	13.8
chronic conjunctivitis	3	5.2
urinary tract infections	5	8.6
Herpes simplex infection	8	13.8
Clinically relevant EBV infection	2	3.4
Pneumonia	13	22.4
Enterocolitis	6	10.3
Pyodermia	3	5.2
Mycoses	4	6.9
Warts	1	1.7
Molluscum contagiosum infection	2	3.4
Tuberculosis	1	1.7
Sepsis	2	3.4
HCV infection	2	3.4
HBV infection	2	3.4
COVID-19	2	3.4
Autoimmune diseases	**9**	**15.5**
neutropenia	4	6.9
psoriasis	1	1.7
arthritis	4	6.9
Malignancies	**35/80**	**43.8**
lymphoma	22	27.5
leukemia	8	10.0
rabdomyosarcoma	1	1.3
ovarian cancer	1	1.3
undefined	3	3.8
Allergies	**5**	**8.6**

positive effect. The patient developed gastrointestinal bleeding and multi-organ failure progressed. The child died on day 253 after transplantation.

Discussion

The diagnosis of NBS in Ukraine began in 1998-2001 at the Department of Hematology of the Lviv Regional Children's Specialized Hospital (now Western Ukrainian Specialized Children's Medical Center) (22, 23). Molecular diagnosis for the first patients was conducted at the Institute of Medical and Human Genetics Charité, Berlin, Germany (3, 7, 24). The molecular-genetic diagnosis of NBS in Ukraine began in 2004 at the Institute of Hereditary Pathology, Lviv.

Professor Larisa Kostyuchenko played a significant role in the study, diagnosis, and treatment of NBS patients in Ukraine (18, 23, 25). The first cohort of patients (1999-2007) were described by Dr. Kostyuchenko in 2009 (18).

The prevalence of NBS patients with the Slavic variant in the western regions of Ukraine as of 2017 was 3.6 per 1,000,000, reaching up to 24 per 1,000,000 in some areas of this region (4), one of the highest in Europe and the world. The highest prevalence of NBS was reported in Poland and the Czech Republic, where it was 3.1 per 1,000,000 (19, 24).

According to the results of genetic testing of the 657del5 variant of the *NBN* gene in randomly selected Gutherie newborn screening cards of healthy newborns, the heterozygous carrier frequency in the Czech Republic was 1:130, in Poland 1:253, and in the Lviv population of Ukraine 1:182 (3). Consequently, the expected frequency of NBS in the Lviv region was 1:133,000 newborns. However, when calculating based on the frequency of verified NBS cases from 2001-2010, it turned out to be significantly higher, at 1:34,000, with heterozygous carriers frequency at 1:95 newborns (20). These results align with the rates observed in the

TABLE 4 Immunoglobulins and lymphocyte subpopulations in NBS patients (n=48).

Parameter	Deviation	n (%)	Me (interquartile range, IQR)	Range (min-max)
Lymphocyte, cells/mm^3	decreased	31 (64.6)	1580 (803; 2310)	38-10100
CD3+, cells/mm^3	decreased	32 (81.8)	847 (510; 985)	190-2510
CD4+, cells/mm^3	decreased	43 (89.6)	407 (295; 475)	60-2050
CD8+, cells/mm^3	decreased	30 (62.5)	284 (116; 491)	96-1072
CD19+, cells/mm^3	decreased	43 (89.6)	106 (78; 180)	10-875
NK, cells/mm^3	increased	30 (62.5)	393 (283; 570)	2-1360
	decreased	2 (4.2)		
Ig G, g/l	decreased	35 (72.9)	3.70 (2.08; 8.01)	0.80 – 14.7
Ig A, g/l	decreased	27 (56.3)	0.13 (0.02; 0.50)	0 – 2.23
Ig M, g/l	increased	10 (20.8)	0.75 (0.41; 1.1)	0 – 2.20
	decreased	4 (8.3)		
Ig E, IU/ml	decreased	18/30 (60)	12 (1; 104)	0.1-1317
	increased	4/30 (13.3)		

northern and eastern regions of Wielkopolska province, Poland (1:76-77) (26) and the vicinity of Novi Sonch, which is geographically close to Lviv (1:90) (3).

However, according to our data, the overall frequency of NBS in Ukraine is 1:141,000 newborns. Overall, our study showed a trend towards improving the diagnosis of NBS over the past 25 years. The increase in the number of diagnosed patients and the shift in the percentage of patients from the western regions of Ukraine to other regions demonstrates improved awareness of NBS among physicians, better accessibility to genetic diagnostics, and population migration. The war may have affected access to diagnostics in eastern and southern regions, which are frontline areas (27), although there is a steady trend towards improved diagnosis in central regions.

The median age of diagnosis has become younger - from 5.5 in 2008-2017 to 2.7 years in 2018-2023. It is worth noting that in 2022, expanded neonatal screening for 21 diseases, including severe combined immunodeficiency (SCID), was introduced in Ukraine. A pilot project of newborn screening using TREC and KREC assay to determine T- and B-lymphopenia confirmed the diagnostic capabilities for NBS (28). It was found 100% sensitivity for TREC copies and 56% sensitivity for KREC copies for the detection of NBS (29). In 2023, NBS was diagnosed in two children due to positive results of newborn screening for SCID confirmed by genetic analysis.

Wolska-Kuśnierz et al. (19) 2015 described 149 NBS patients with the Slavic variant of *NBN* from the European Registry for PID, among which the majority were patients from Poland (118) and Ukraine (14). Overall, the median age of NBS patients in our cohort is slightly lower than in the mentioned study (11 versus 14.3 years). The percentage of deceased patients in our study is slightly lower (33.3% versus 39%), but the children died at a younger age (median age of deceased 9 years versus 11.1 years).

Overall, 6.9% of patients were born prematurely, but 70.7% had low birth weight (less than 3000g), while a body length of less than 50 cm was observed in 43.1% of children. Our results coincide with the data described in previous studies (6, 19). Although there were no premature births in the previous cohort, 42.9% of mothers experienced spontaneous abortions during previous pregnancies (18).

Data reflecting the dynamics of physical development in NBS are limited. We have shown that up to the age of 10, most patients lag in weight, and a significant portion (from 28.6% to 50%) in body length/height. Another study also showed significantly lower anthropometric indicators in NBS patients than in the healthy population (11). As in our observations, Varon et al. (6) also note that growth deficiency improves with age. The improvement in the physical condition of children with NBS may be associated with better diagnosis as well as improvements in socio-economic status, treatment options for NBS, and associated conditions that arise with it. Microcephaly was observed in all patients with a median deviation in head circumference of -6.5z at 1 year.

Our data related to infections differ slightly in some indicators from the results described by Wolska-Kuśnierz et al. in 2015 (19). Their study observed recurrent infections without severe course or complications in 23% of patients, but 43% experienced severe respiratory problems. According to our study, a lower frequency of pneumonia (22.4% versus 46.4%), chronic bronchitis, and bronchiectasis (6.9% versus 23.6%) was also noted, but recurrent sinusitis (17.2% versus 8.2%) and recurrent labial herpes infection (13.8% versus 0.9%) were more common. Data on SARS-CoV-2 infection in three patients showed a mild course, which in one patient was complicated by arthritis three weeks after COVID-19 (29, 30). The decrease in the frequency of recurrent respiratory infections in recent years may be associated with both earlier NBS diagnosis and better access to immunoglobulin replacement

therapy, as well as patient isolation during the COVID-19 pandemic. It should be noted that infections were the cause of death in three cases (12% of deaths), which coincides with the results of previous studies, where 14% of patients died from pulmonary infections with respiratory insufficiency (19).

Malignancies were diagnosed in 43.8% of children, which aligns with the results of other studies where oncopathology was diagnosed in 42% (19) and 45% (4) of NBS patients. Malignancies were more common in boys (p=0.0399), which is consistent with the results of another study (4). Lymphomas predominated in patients with malignancies (62.9%), as reported in previous studies (4, 19, 22). Among lymphomas, aggressive DLBCL type prevalated (45.5%). The median age of children at the time of malignancy diagnosis was 9.5 years, which also corresponds to the results of other studies (19, 23).

Autoimmune and allergic manifestations are also characteristic of IEI (31, 32), although they were less common in NBS patients (15.5% and 8.6%, respectively), which aligns with reports from other researchers (19).

The analysis of immunological changes in the presented cohort of patients mostly corresponded to those changes described previously (19, 33). The number of NK cells was increased in the majority of NBS patients. Another study indicated that in most patients, the number of NK cells was within the normal range (79%), with elevated levels in 13% and decreased levels in 8% (19). However, the study of Gregorek et al. (33) confirmed our results, as the number of NK cells in NBS patients was significantly higher than in healthy controls (32.3% vs. 9.7%, $p < 0.01$) (33). Researchers also demonstrated significant deviations in the maturation process of peripheral T lymphocytes in NBS patients compared to healthy individuals. The substantial increase in populations of effector T cells in NBS patients may be associated with increased susceptibility to malignancies and a milder clinical course than expected, considering T-cell lymphopenia (34). Researchers did not find a correlation between T-cell lymphopenia and the frequency and severity of respiratory infections (19). It was also reported that abnormalities may progress over time (33); however, this hypothesis lacks credible evidence.

Changes in humoral immunity were most commonly reflected in decreased levels of IgG (72.9%) and IgA (56.3%), which also corresponds to the data from previous studies (62% and 57%, respectively) (19). The authors noted a correlation between the degree of IgG and IgA deficiency and the severity and/or chronicity of respiratory tract infections (19).

The treatment of NBS patients remains challenging due to the lack of standardized therapeutic options. In all cases, the degree of immunodeficiency should be assessed, and any infectious complications require prompt decision. Immunoglobulin replacement therapy allows control for the infectious syndrome in the majority of patients. These patients respond poorly to treatment of malignancy due to radiosensitivity and impaired tolerance to chemotherapy. Allo-HSCT may be considered as a treatment option aimed at correcting the immune system. However, it is still controversial and requires further research, for the optimal preparative regimen before HSCT, graft sources, choice of graft-versus-host disease prophylaxis method, peculiarities of pre- and post-transplantation support, and so on (21, 35).

Thus, the conducted study revealed the wide prevalence of NBS in Ukraine and a tendency towards improving its diagnosis. However, considering the high carrier frequencies of NBN in Czech, Ukrainian, and Polish populations (3), the number of patients in Ukraine should be higher, indicating a significant number of undiagnosed cases. Increasing awareness among the medical community and raising public awareness may improve the diagnosis of IEI, including NBS in Ukraine (36, 37). Implementing newborn screening for T- and B-cell lymphopenia may also help improve early diagnosis of NBS, as well as careful registration and vigilance regarding patients with microcephaly, growth delay, and malignancy. Prenatal diagnosis is important for families with cases of children's death at an early age from severe or recurrent infections or malignancies, and the presence of individuals in the family with microcephaly. Frequent miscarriages should also raise suspicion of NBS. Considering the high frequency of the 657del5 variant of the *NBN* gene, predictive genetic testing may be considered. Early diagnosis will allow for appropriate prevention of infections, avoiding radiation exposure for diagnosing infections and other pathologies, and selecting the appropriate strategy for treating malignancies.

Strengths and limitations of the study

A noteworthy aspect of this study is that it is the first comprehensive analysis of the prevalence, clinical, and immunological characteristics of patients from various regions of Ukraine over 25 years. Previous studies mainly focused on data from patients in western regions during the initial 10 years of diagnosis, or the prevalence of malignancies was limitedly studied. The cohort of Ukrainian patients with Slavic variant in the *NBN* gene is one of the largest in the world which enhances the generalizability of the findings. The analysis was conducted on the growth and weight parameters of children with NBS from birth to 18 years, which has been insufficiently addressed in previous publications.

However, a limitation of this study is its retrospective data collection, which posed challenges in obtaining certain data, as well as the possibility of missing data or inaccuracies in the medical records and conducting a thorough assessment of result completeness. Transitioning to adult care resulted in worsened patient monitoring, making it difficult to track data. Another limitation of this study is that we identified the c.657_661del5 variant of the *NBN* gene, while around 10% of patients with NBS harbor alternative pathogenic variants, which could influence the epidemiological results. The prevalence of NBS in the Ukrainian population may be higher with improved diagnosis and surveillance. Nonetheless, analyzing data longitudinally allows for identifying diagnostic trends and determining ways to improve both diagnosis and the management of NBS patients.

Conclusion

The cohort of diagnosed NBS patients with the 657del5 variant in the *NBN* gene in Ukraine currently comprises 84 individuals, making it one of the largest in Europe and the world. Most patients originate from western regions of Ukraine (57.8%), although in recent years, there has been an increase in diagnoses from central and southeastern regions, expanding our knowledge of NBS prevalence. Malignancies have been the most common cause of death in NBS patients, accounting for 72% of cases. Regular prophylactic immunoglobulin replacement therapy has helped reduce the frequency and severity of severe respiratory tract infections.

Improvements in diagnosis, including prenatal screening, newborn screening, monitoring, and the expansion of treatment options, will lead to better outcomes for NBS patients.

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 humans were approved by I.Horbachevsky Ternopil National Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

OBo: Conceptualization, Data curation, Formal analysis, Project administration, Software, Supervision, Writing – original draft, Writing – review & editing. LK: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. HA: Visualization, Writing – original draft, Writing – review & editing. ABo: Data curation, Writing – original draft, Writing – review & editing. AV: Writing – original draft, Writing – review & editing, Data curation. AH: Data curation, Writing – original draft, Writing – review & editing. IS: Writing – original draft, Writing – review & editing. LN: Writing – original draft, Writing – review & editing. NY: Writing – original draft, Writing – review & editing. OU: Writing – original draft, Writing – review & editing. IH: Writing – original draft, Writing – review & editing. OL: Writing – original draft, Writing – review & editing. ABu: Writing – original draft, Writing – review & editing. OT: Writing – original draft, Writing – review & editing. MI: Writing – original draft, Writing – review & editing. OBa: Writing – original draft, Writing – review & editing. EP: Writing – original draft, Writing – review & editing. NV: Writing – original draft, Writing – review & editing. YS: Writing – original draft, Writing – review & editing. LC: Writing – original draft, Writing – review & editing. HM: Investigation, Methodology, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

This article is dedicated to the memory of LK, who passed away at the end of 2023 and devoted her entire professional life to diagnosing and treating patients with primary immunodeficiencies. One of her significant contributions was the study of NBS. She first described the clinical and immunological manifestations in a cohort of Ukrainian patients with NBS. LK began work on an article where she wanted to gather all accumulated experience regarding the diagnosis and treatment of this syndrome in Ukraine. We also want to thank the Institute of Hereditary Pathology scientists, currently headed by HA, in particular to Yuriy Havrylyuk, Aleksey Kashin, HM, Nataliya Huleyuk, Halyna Bezkorovayna, Nataliya Markevych, Nataliya Kitsera and all staff members. We express our gratitude to the oncologists and hematologists of the Western Ukrainian Specialized Children's Medical Center, its former head Romana Polishchuk who first paid attention to children with oncopathology and characteristic appearance, thereby drawing attention to the problems of patients with NBS. We also want to thank all immunologists and oncologists who manage and treat NBS patients in Ukraine. Special thanks to the specialists of the Institute of Medical and Human Genetics Charité (Berlin, Germany) and the Department of Medical Genetics, The Children's Memorial Health Institute (Warsaw, Poland) for their assistance in diagnosing the first patients and establishing genetic diagnostics in Ukraine.

Conflict of interest

Author HM is employed by Scientific Medical Genetic Center LeoGENE, LTD.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 12 July 2024
DOI 10.3389/fimmu.2024.1402038

OPEN ACCESS

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RECEIVED 16 March 2024
ACCEPTED 27 June 2024
PUBLISHED 12 July 2024

CITATION
Tahiat A, Belbouab R, Yagoubi A, Hakem S, Fernini F, Keddari M, Belhadj H, Touri S, Aggoune S, Stoddard J, Niemela J, Zerifi F, Melzi S, Aboura R, Saad-Djaballah A, Ferhani Y, Ketfi A, Messaoudi H, Bencharif Madani T, Benhacine Z, Dehimi A, Okka K, Amroune F, Fellahi M, Bendahmane C, Khoulani R, Oukil A, Soufane A, Bourelaf I, Boubidi C, Boukhenfouf N, Amine Ifri M, Khelafi N, Boudiaf H, Khelifi Touhami T, Meçabih F, Boucelma M, Zelaci A, Gacem O, Ladj MS, Mekki A, Bensaadi N, Benhalima M, Zeroual Z, Bioud B, Benameur M, Bouhdjila R, Bouzerar Z, Ibsaine O, Maouche H, Kedji L, Smati L, Boukari R, Lambert C, Rosenzweig SD, Notarangelo LD and Djenouhat K (2024) Flow cytometry-based diagnostic approach for inborn errors of immunity: experience from Algeria. *Front. Immunol.* 15:1402038. doi: 10.3389/fimmu.2024.1402038

Flow cytometry-based diagnostic approach for inborn errors of immunity: experience from Algeria

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Purpose: In this study, we retrospectively reviewed the use of flow cytometry (FCM) in the diagnosis of inborn errors of immunity (IEIs) at a single center in Algeria. Sharing insights into our practical experience, we present FCM based diagnostic approaches adapted to different clinical scenarios.

Methods: Between May 2017 and February 2024, pediatric and adult patients presenting with clinical features suggestive of immunodeficiency were subjected to FCM evaluation, including lymphocyte subset analysis, detection of specific surface or intracellular proteins, and functional analysis of immune cells.

Results: Over a nearly seven-year period, our laboratory diagnosed a total of 670 patients (372 (55.5%) males and 298 (44.5%) females), distributed into 70 different IEIs belonging to 9 different categories of the International Union of Immunological Societies classification. FCM was used to diagnose and categorize IEI in 514 patients (76.7%). It provided direct diagnostic insights for IEIs such as severe combined immunodeficiency, Omenn syndrome, MHC class II deficiency, familial hemophagocytic lymphohistiocytosis, and CD55 deficiency. For certain IEIs, including hyper-IgE syndrome, STAT1-gain of function, autoimmune lymphoproliferative syndrome, and activated PI3K delta syndrome, FCM offered suggestive evidence, necessitating subsequent genetic testing for confirmation. Protein expression and functional assays played a crucial role in establishing definitive diagnoses for various disorders. To setup such diagnostic assays at high and reproducible quality, high level of expertise is required; in house reference values need to be determined and the parallel testing of healthy controls is highly recommended.

Conclusion: Flow cytometry has emerged as a highly valuable and cost-effective tool for diagnosing and studying most IEIs, particularly in low-income countries where access to genetic testing can be limited. FCM analysis could provide direct diagnostic insights for most common IEIs, offer clues to the underlying genetic defects, and/or aid in narrowing the list of putative genes to be analyzed.

KEYWORDS

flow cytometry, lymphocyte phenotyping, protein expression assays, functional assays, flow cytometry-based diagnostic approach, complement deficiencies

1 Introduction

Human inborn errors of immunity (IEIs) are a heterogenous group of monogenetic disorders characterized by an absent or aberrant function in one or more components of the immune system, which predisposes affected individuals to increased frequency and severity of infection, autoimmunity, allergy, and malignancy (1, 2). IEIs were traditionally viewed as rare disorders. However, recent reports suggest that IEIs are more common than previously believed, with a collective prevalence ranging from 1/5000 to 1/1000 (3). Furthermore, the wider usage of next-generation sequencing (NGS) platforms had greatly contributed to the discovery of new IEIs and broadened the spectrum of clinical phenotypes associated with IEI (4, 5).

The latest report from the International Union of Immunological Societies (IUIS) Expert Committee described 485 different disorders, categorized into ten groups (1). Most disorders in the IUIS classification have an autosomal recessive (AR) mode of inheritance, suggesting IEIs should be even more prevalent in consanguineous populations from North African countries like

Algeria. However, the reported prevalence of ~1/50,000 in the Algerian registry remains low (6, 7). This means there is a significant underestimation of the burden of IEIs in Algeria. Several factors are likely culprits: insufficient awareness, a high frequency of severe forms–especially combined immunodeficiencies –linked to early mortality, and limitations in the availability of necessary diagnostic tools (6, 8).

The basic clinical and laboratory evaluation of a patient with a suspected IEI should encompass a detailed clinical history, a complete blood count (CBC), measurements of serum immunoglobulin (Ig) levels, and complement protein assays (9). Due to the heterogeneity of the clinical presentations of patients, diagnosing IEIs based solely on clinical and conventional laboratory findings can often be challenging. While more recently available genetic investigations serve as definitive tool for diagnosing IEIs, genetic tests are time-consuming, labor-intensive, and costly. Additionally, targeted single-gene tests may overlook causative mutations. Exome sequencing has recently garnered attention as a gold standard for diagnosing IEIs. However, exome sequencing still identifies causative genetic defects in only 40% to 58% of patients (10, 11). Moreover, establishing such a facility in the context of a developing country is highly challenging. Therefore, flow cytometry (FCM) emerges as a valuable and cost-effective tool bridging conventional laboratory testing and genetic testing. It provides a rapid and accurate results based on single-cell analysis (12).

Several authors have described the role of FCM in the diagnosis of IEIs; however, few have presented experiences from specific centers, and none from the Maghreb region (13–15). In this study, we retrospectively reviewed the use of FCM in the diagnosis of IEIs at a single center in Algeria, emphasizing the experiences of a diagnostic approach adapted to various clinical scenarios.

2 Materials and methods

The study was conducted at the department of medical biology in Rouiba hospital and received ethical approval from the local committee in accordance with the Declaration of Helsinki. The study was designed as a retrospective review of FCM's use in diagnosing IEIs over a nearly seven-year period, from May 2017 to February 2024. All patients met the updated criteria of the European Society for Immunodeficiency (ESID) (www.esid.org) and were categorized according to the updated classification of the IUIS expert committee (1).

2.1 Patients' enrollment and data collection

Peripheral blood samples were received from both pediatric and adult patients presenting with clinical features suggestive of immunodeficiency, such as repeated, unusual, or severe infections, as well as early-onset immune dysregulation conditions, including cytopenia, inflammatory bowel disease, and endocrinopathy. These samples were referred to our center from various pediatric, pneumology, internal medicine and infectious diseases departments affiliated with various public healthcare facilities spread across the whole country. Data were systematically collected for each patient using a standardized data form and entered into a computerized database. Requested information included patient's demographics, family history, clinical history (including the age of onset and the main infectious and/or immune dysregulation manifestations; thanks to the close collaboration with clinicians), and laboratory and imaging findings.

2.2 Initial laboratory evaluation

Before delving into the exploration of primary immunodeficiency, we meticulously excluded the presence of any secondary immunodeficiencies, with a particular focus on HIV infection, as well as those induced by the use of drugs or renal and gastrointestinal losses. In addition to routine laboratory parameters, including complete differential blood counts, peripheral blood smear, serum immunoglobulin measurements, and complement protein assays, the initial assessment of IEI also included a basic immunophenotyping of circulating lymphocyte subsets, referred to as T-B-NK enumeration. This assay utilized six different markers to analyze various lymphocyte subsets, including CD3 as a pan-T marker, CD4 for T-helper cells, CD8 for T-cytotoxic cells, CD19 for B cells, and CD56/CD16 for NK cells. Lymphocytes were gated using CD45 *vs.* side scatter (SSc), and the percentages of different subpopulations were determined. FCM analysis were performed in a six-colors FACSCanto™ cytometer (BD Biosciences, US.) from 2017 to 2021 and in an eight color FACSLyric™ cytometer (BD Biosciences, US.) from 2022 to date.

2.3 Advanced flow cytometry evaluation

More advanced FCM analysis was performed when needed, based on clinical presentation (e.g., age of onset, location, types of infectious and autoimmune/inflammatory diseases etc.) and conventional laboratory findings. These specialized FCM tests include (Table 1):

i. Extended phenotyping of circulating T- and B-cell subsets;
ii. Analysis of the expression of specific surface or intracellular proteins;
iii. Functional assays, including cytokine production, STAT1 and STAT3 phosphorylation, and NK-cell degranulation assays.

Detailed methods can be found in this article's Online Repository at www.frontiersin.org.

2.3.1 Extended phenotyping of T- and B-cell subsets

2.3.1.1 Extended T-cell phenotyping

A detailed analysis of naive and memory T-cell subpopulations was conducted in all patients with T lymphopenia and/or an initial

10.3389/fimmu.2024.1402038

TABLE 1 Lymphocyte phenotyping, disease specific protein analysis and functional assays for IEI diagnosis.

Test	Cell populations	Indications
Immunophenotyping of circulating lymphocytes		
Basic T-B-NK phenotyping	CD4+ T cells, CD8+ T cells, B cells, NK cells	Basic screening for IEI, CID, SCID, PAD
Extended T-cell phenotyping		
T1 panel	naïve ($CD45RA^+CCR7^+$), memory ($CD45RO^+$) and CD8+ TEMRA ($CD45RA^+CCR7^-$)	SCID, OS, leaky SCID, CID, CVID
T2 panel	RTE ($CD4^+CD45RA^+CD31^+$)	SCID, OS, leaky SCID, DiGeorge syndrome
T3 panel	Th1 ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$), Th17 ($CXCR3^-CCR6^+$), Tfh ($CXCR5^+$)	HIES, CMC, CID
T4 panel	TCRαβ DNT ($CD3^+TCR\alpha\beta^+CD4^-CD8^-$)	ALPS, Patients with >7% of CD3+CD4-CD8- T cells
T5 panel	Treg ($FoxP3^+CD25^+$)	IPEX syndrome
Extended B-cell phenotyping		
B panel	switched memory ($CD27^+sIgD^-$), non-switched memory ($CD27^+sIgD^+$), transitional B cells ($CD38^{hi}CD24^{hi}$), plasmablasts ($CD38^{hi}CD24^-$) and $CD21^{lo}$ B cells ($CD38^{low}CD21^{low}$)	CVID, HIMS, PAD, CID, LRBA deficiency, RIPK1 deficiency
Analysis of the expression of specific surface or intracellular proteins		
CD132	B cells	$T^-B^+NK^-$ SCID
CD127	T cells	$T^-B^+NK^+$ SCID
HLA-DR	B cells	CID with low CD4 (MHC-II deficiency)
HLA-ABC	lymphocytes	CID with low CD8 (MHC-I deficiency)
ZAP-70	T cells	CID with low CD8
CD40L	PMA/Ionomycin stimulated CD4+ T cells	X-linked HIMS
CD40	B cells	HIMS
WASp	T cells	WAS
Btk	monocytes	XLA
Perforin	NK cells	FLH 2
CD18/CD11a	neutrophils	LAD-1
CD15	neutrophils	LAD-2
IFN-γR1 (CD119)	monocytes	MSMD
IL-12Rβ1 (CD212)	PHA stimulated T cells	MSMD
MCP/CD46	lymphocytes	aHUS
DAF/CD55	neutrophils	PLE (CHAPLE disease)
Functional assays		
Oxidative burst assay (DHR 123 assay)	neutrophils	CGD, EO-IBD
IL-17/IFN-γ production	PMA/Ionomycin stimulated CD4+ T cells	HIES, CMC
STAT3/STAT1 phosphorylation assays	IL6/IFN-γ stimulated T cells/monocytes	HIES, MSMD, CMC
NK-cell degranulation assay	PMA/Ionomycin/Brefeldin stimulated $CD3^-CD56^+$ NK cells	FLH 3, 4 and 5, CHS, GS2

aHUS, atypical hemolytic uremic syndrome; ALPS, autoimmune lymphoproliferative syndrome; Btk, Bruton's tyrosine kinase; CGD, chronic granulomatous disease; CID, combined immunodeficiency; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CHS, Chediak-Higashi syndrome; CMC, chronic mucocutaneous candidiasis; CVID, common variable immunodeficiency; DAF, decay accelerating factor; DHR, Dihydrorhodamine; EO-IBD, early onset inflammatory bowel disease; FHL, familial hemophagocytic lymphohistiocytosis; GS2, Griscelli syndrome type 2; HIMS, hyper-IgM syndrome; HIES, hyper-IgE syndrome; HLA, human leukocyte antigen; IEI, inborn errors of immunity; IPEX, immune dysregulation-polyendocrinopathy-enteropathy-x-linked; LRBA, LPS-responsive beige-like anchor protein; LAD, leukocyte adhesion deficiency; MCP, membrane cofactor protein; MHC, major histocompatibility complex; MSMD, mendelian susceptibility to mycobacterial disease, OS, Omenn syndrome, PAD, predominantly antibody deficiency; PHA, phytohemagglutinin; PLE, protein losing enteropathy; PMA, phorbol-12-myristate-13-acetate; RIPK1, receptor-interacting serine/threonine-protein kinase 1; RTE, recent thymic emigrants; SCID, severe combined immunodeficiency; sIgD, surface IgD; TEMRA, terminally differentiated effector memory CD45RA+; Treg, regulatory T cells; WAS, Wiskott Aldrich syndrome; ZAP-70, Zeta-associated protein 70.

clinical and laboratory evaluation suggestive of Omenn syndrome (OS), atypical severe combined immunodeficiency (SCID) or CID. A six-color panel (T1 panel) was used for this purpose, incorporating CD3, CD4, CD8, CD45RA, CD45RO, and CCR7 (CD197) markers, enabling the identification of naïve (CD45RA +CCR7+), memory (CD45RO+), and terminally differentiated effector memory CD45RA+ (TEMRA) among CD4+ or CD8+ T cells (Table 1, Supplementary Table S1). In some cases, CD31 (PECAM-1) was used as a marker to aid in the identification of CD4+ recent thymic emigrants (RTE) expressing CD45RA and CD31 markers (T2 panel) (16, 17).

In addition, other panels have been designed to identify further specific T-cell subpopulations (Supplementary Table S1). In patients with suspected hyper-IgE syndrome (HIES), Th17 cells were evaluated by a surface staining of memory CD4+ T cells (gated on CD45RO *vs.* CD4 dot plot), measuring membrane expression of CXCR3 (CD183) and CCR6 (CD196) chemokine receptors (T3 panel). Th17 cells exhibit the following profile: $CD45RO^+CXCR3^-CCR6^+$ (18). TCRαβ double-negative T cells (TCRαβ DNT) were estimated in patients with suspected autoimmune lymphoproliferative syndrome (ALPS) or elevated $CD3^+CD4^-CD8^-$ T cells, using the flowing panel: TCRαβ, TCRγδ, CD3, CD4, and CD8 (T4 panel).

Regulatory T cells (Treg) were identified either by the analysis of intracellular FoxP3 expression of or by surface staining of CD4, CD45RA, CD25, CD127 markers in suspected cases of Immune Dysregulation, Polyendocrinopathy, Enteropathy X-Linked (IPEX) Syndrome (T5 and T5bis panel).

2.3.1.2 Extended B-cell phenotyping

Extended phenotyping of circulating B cells was performed in case of suspected predominantly antibody deficiency (PAD) with normal to moderately decreased B cells (B cells >2%). A multicolor panel, incorporating CD19, CD27, sIgD, CD24, CD38, and CD21 markers was used to analyze different B-cell subsets, including naïve ($CD27^{-}sIgD^{+}$), switched memory ($CD27^{+}sIgD^{-}$), non-switched memory ($CD27^{+}sIgD^{+}$), transitional ($CD38^{hi}CD24^{hi}$), plasmablasts ($CD38^{hi}CD24^{-}$) and CD21-low ($CD21^{lo}$) B cells ($CD38^{low}CD21^{low}$) (Supplementary Table S1).

2.3.2 Analysis of the expression of specific surface or intracellular proteins

2.3.2.1 Cell surface proteins

Flow cytometry was used to evaluate specific cell surface proteins, targeted based on the suspected diagnosis (Table 1). Both percentage and mean/median fluorescence intensity (MFI) were determined and compared to controls. Common γ chain (CD132) and IL-7RA (CD127) expression were evaluated in patients with suspected X-linked (XL)-SCID (T-B+NK-SCID) and T-B+NK+SCID, respectively. Patients with suspected CID were systematically screened for major histocompatibility complex class II (MHC-II) deficiency by the assessment of human leukocyte antigen-DR (HLA-DR) on B cells and monocytes. MHC class I expression on lymphocytes was performed in patients with isolated CD8 lymphopenia. For patients with suspected hyper-IgM syndrome (HIMS), we studied CD40 expression on B cells (AR-HIMS) and CD40L (CD154) expression on CD4+ T cells after stimulation with phorbol myristate acetate (PMA) and ionomycin (X-linked HIMS). FCM-based diagnosis of LAD-1 and LAD-2 included the assessment of CD18/CD11a (LAD-1) and CD15 (LAD-2) expression on gated neutrophils. IFN-γR1 (CD119) and IL-12Rβ1 (CD212) were analyzed in patients with a picture suggestive of mendelian susceptibility to mycobacterial disease (MSMD). Patients with atypical hemolytic uremic syndrome (aHUS) and early-onset protein-losing enteropathy (PLE) were screened for CD46/MCP (membrane cofactor protein) deficiency and CD55/DAF (decay accelerating factor) deficiency, respectively (Table 1).

2.3.2.2 Intracytoplasmic proteins

Selected intracytoplasmic proteins were analyzed on permeabilized lymphocytes or monocytes according to the suspected diagnosis (Table 1). Isotypic controls (IC) were used to calculate a staining index, defined as the ratio of the MFI of the targeted protein to that of the IC. The staining index was determined for both patients and controls. In patients with suspected Wiskott–Aldrich syndrome (WAS), intracytoplasmic staining for WAS protein (WASp) was performed using purified mouse anti-human WASp monoclonal antibody and secondary FITC-conjugated anti-Mouse IgG2a. WASp expression was evaluated on T cells. ZAP-70 staining on T cells was performed in patients with isolated CD8 lymphopenia. For male patients with suspected X-Linked Agammaglobulinemia (XLA), Bruton's tyrosine kinase (Btk) protein expression analysis was carried out on permeabilized CD14+ monocytes. Patients with hemophagocytic lymphohistiocytosis (HLH) were screened for perforin deficiency (i.e., Familial hemophagocytic lymphohistiocytosis type 2 or FHL2) by analyzing the expression of perforin on permeabilized $CD3^{-}CD56^{+}$NK cells.

2.3.3 Functional assays

2.3.3.1 Dihydrorhodamine 123 assay

DHR 123 assay was used for the diagnosis of chronic granulomatous disease (CGD). Patient's neutrophils were stimulated for 20 minutes with PMA in the presence of DHR 123. A stimulation index (SI) was calculated for gated neutrophils (SI: the ratio of the MFI of the stimulated to the unstimulated neutrophils). SI of 100 was considered the cutoff in our laboratory.

2.3.3.2 Cytokines production assay

In patients with picture of HIES or chronic mucocutaneus candidiasis (CMC), IL-17A and IFN-γ production was measured following stimulation of whole blood or peripheral blood mononuclear cells (PBMC) with PMA/Ionomycin in the presence of protein transport inhibitor (Brefeldin).

2.3.3.3 Signal transducers and activators of transcription phosphorylation assays

STAT3 phosphorylation was evaluated on CD4+ T cells after stimulation of PBMC with IL-6 followed by fixation and permeabilization of the cells and incubation with anti-pSTAT3. A SI was calculated for gated CD4+ T cells by dividing the MFI of stimulated cells by the MFI of unstimulated cells. STAT1 phosphorylation after IFN-γ stimulation was evaluated in patients with suspected MSMD or CMC due to gain of function (GOF) mutation in STAT1.

2.3.3.4 NK-cell degranulation assay

The degranulation assay of resting NK cells was conducted in patients with HLH and normal perforin expression, along with those suspected of Chediak–Higashi syndrome (CHS) or Griscelli syndrome type 2 (GS2). After two hours stimulation of whole blood or PBMC with PMA and ionomycin, $CD3^{-}CD56^{+}$NK cells were gated and assessed for surface expression of CD107a. ΔCD107a, which is the difference of surface CD107a expression between stimulated and non-stimulated NK cells, was determined. Defective degranulation was arbitrarily defined as less than 5% degranulation, whereas abnormal degranulation was defined as being lower than 10%.

2.4 Genetic testing

Genetic testing is not routinely available in our laboratory. When feasible, patients with MHC-II deficiency underwent screening for the 752delG26 mutation (deletion of 26bp) by analyzing the size of PCR products by electrophoresis in a 2% agarose gel. Sanger sequencing was performed on specific genes including, *RAG1*, *RAG2*, *ADA*, *IL2RG*, *CD40, DOCK8*, *WAS, BTK,*

LYST, *ITGB2, C3, and CD46*. Next generation sequencing (NGS) through targeted gene sequencing panels or whole exome/genome sequencing was performed in patients with undefined clinical and/or immunological picture.

3 Results

3.1 Baseline characteristics

Between May 2017 and February 2024, a total of 4,277 patients underwent screening for IEI at our center. Among them, 670 patients, comprising 372 (55.5%) males and 298 (44.5%) females, were diagnosed with IEI. The median age of IEI patients at time of diagnosis was 6 years (0.1 – 81 years), and the median age at the onset of symptoms was 1 year (0 – 66 years). Four hundred eighty-seven patients (72.7%) were children, while 183 (27.3%) were adults. Nearly half of patients (47.5%) were born from consanguineous parents. FCM helped to categorize and reach the diagnosis in 514 patients (76.7%), while 156 (23.3%) were categorized based on other laboratory parameters (e.g., measurement of serum complement C4 and C1-inhibitor in hereditary angioedema), clinical manifestations and/or genetic testing (Supplementary Table S2). Of the 514 patients, flow cytometry analysis yielded a definitive diagnosis in 104 cases. For the remaining patients, flow cytometry proved instrumental in categorizing IEI into functional groups and provided valuable insights into the underlying genetic defect, albeit requiring subsequent genetic testing for confirmation (Figure 1). Genetic analysis was performed in 118 patients, revealing the genetic defect in 93 (78.8%) patients. The distribution of patients according to the classification from the IUIS expert committee is shown in Table 2 and Supplementary Figure S1. Combined immunodeficiencies (24.3%) were the most common, followed by predominantly antibody deficiencies (23.1%) and complement deficiencies (22.8%). Hereby we present the results of the patients screened and/or diagnosed based on FCM results.

3.2 Combined immunodeficiencies

Combined immunodeficiencies were the most common with 163 cases, including 30 (18.4%) SCID, 8 (4.9%) Omenn syndrome, 9 (5.5%) leaky SCID, and 35 (21.5%) MHC class II deficiency (Table 2). The diagnosis was made based on FCM analysis in all patients; genetic analysis was carried out in 54 (33.1%) patients, leading to the identification of the genetic defect in 40 (24.5%) patients (Table 3).

3.2.1 Severe combined immunodeficiencies

Of the 30 patients with SCID, 14 (46.7%) were male and 16 (53.3%) were females. The median age at diagnosis was 5 months (range: 2 – 17) and the median age at symptoms onset was one month (range: 0.1 – 4). All but one patient had less than 300 T cells/μl and the mean T-cell count was 58/μl (range: 0 – 545). Twenty-one (70%) were diagnosed with T-B-NK+ SCID, 5 (16.7%) with T-B+NK+ SCID, 3 (10%) with T-B+NK- SCID, and one (3.3%) with T-B-NK- SCID. Common γ chain expression was evaluated in two male patients with T-B+NK-SCID and was defective in one of them. IL-7RA expression was assessed in one T-B+NK+ SCID patient and was normal. Sanger sequencing was performed based on FCM classification in 5 patients revealing homozygous *RAG1*/*RAG2* variants in 3 patients, and homozygous *ADA* mutation and hemizygous *IL2RG* variant in the remaining two patients (Table 3).

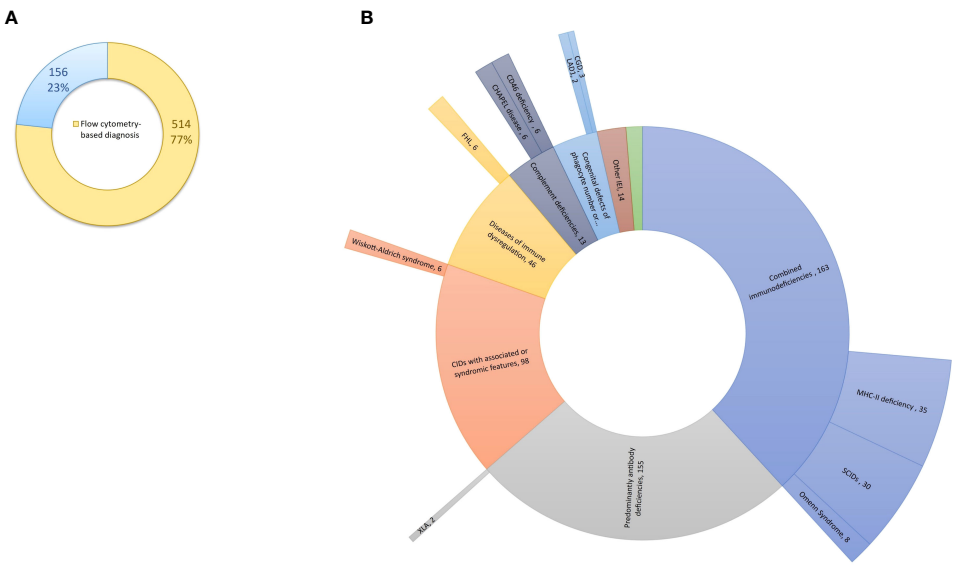

FIGURE 1
Flow cytometry-based diagnosis of IEI at our center. **(A)** Flow cytometry has proven instrumental in diagnosing and categorizing IEI in 514 patients (77%). **(B)** Among the 514 patients (internal circle), flow cytometry analysis led to the definitive diagnosis in 104 cases (external circle).
IEI, inborn errors of immunity.

10.3389/fimmu.2024.1402038

TABLE 2 Distribution and laboratory findings of IEIs in our series.

Category	Disease	N (%)	Age at diagnosis (months) median (range)	Associated flow cytometry findings	
				Findings	*%*
Combined immunodeficiencies		163 (24.3%)	17 (1 – 552)		
	SCIDs	30 (4.5%)	5 (2 – 17)	T cells < 300/µl	96.7%
	Omenn Syndrome	8 (1.2%)	4.5 (1 – 10)	Absent naïve T cells Absent RTE Absent B cells	100% 100% 100%
	Leaky SCIDs	9 (1.3%)	19 (5 – 240)	Decreased naïve T cells Very low RTE Reduced B cells	100% 100% 88.9%
	MHC-II deficiency	35 (5.2%)	20 (1 – 132)	Absent HLA-DR expression on B cells CD4 lymphopenia Inverted CD4/CD8 ratio	100% 88.6% 74.3%
	Other CID	81 (49.7%)	36 (2 – 552)	CD4 lymphopenia Inverted CD4/CD8 ratio Decreased naïve CD8 T cells Decreased naïve CD4 T cells	74.1% 60.5% 95.8%* 81.9%*
CIDs with associated or syndromic features		101 (15.1%)	48 (1 – 204)		
	DiGeorge syndrome	7 (1.0%)	3 (2 – 11)	CD3 lymphopenia	100%
	Ataxia Telangiectasia	11 (1.6%)	90 (30 – 204)	CD3 lymphopenia Decreased naïve T cells	100% 100%
	Wiskott-Aldrich syndrome	16 (2.4%)	8 (1 – 48)	CD4 lymphopenia Inverted CD4/CD8 ratio Absent/reduced WASp expression	62.5% 62.5% 66.7%**
	Hyper-IgE syndrome	57 (8.5%)	60 (8 – 192)	Defective IL-17 production Low $CXCR3^{-}CCR6^{+}$ Th17 cells Absent/reduced STAT3 phosphorylation	81.8%# 45.5%# 9.1%#
	ARPC1B deficiency	2 (0.3%)	96 (72 – 120)	Hyper-IgE CD3 lymphopenia Decreased naïve T cells Expanded TCRαβ DNT	100% 100% 100% 50%
Predominantly antibody deficiencies		155 (23.1%)	96 (5 – 804)		
	Agammaglobulinemia	17 (2.5%)	36 (5 – 312)	Severe decrease of sIg Absent/profoundly decreased B cells Absent Btk expression (in male patients)	100% 100% 100%¥
	CVID	70 (10.4%)	252 (18 – 804)	Reduced switched memory B cells Expanded transitional B cells Expanded $CD21^{lo}$ B cells	80.9%§ 23.8%§ 28.6%§
	Activated p110δ syndrome	2 (0.3%)	21 (18 – 24)	Reduced switched memory B cells Expanded transitional B cells Decreased naïve T cells	100% 100% 100%
	SIAD	15 (2.2%)	72 (30 – 624)		
	Unclassified antibody deficiency	53 (7.9%)	48 (5 – 708)		
Diseases of immune dysregulation		49 (7.3%)	48 (1 – 336)		

(Continued)

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TABLE 2 Continued

Category	Disease	N (%)	Age at diagnosis (months) median (range)	Associated flow cytometry findings	
				Findings	*%*
	Chediak-Higashi Syndrome	9 (1.3%)	30 (1 – 84)	Defective NK-cell degranulation assay	100%[£]
	Griscelli Syndrome	1 (0.1%)	72		
	FHL	7 (1.0%)	14 (1 – 54)	Defective/abnormal NK-cell degranulation	71.4%
	ALPS	5 (0.7%)	42 (10 – 132)	Increased TCRαβ DNT	100%
	RIPK1 deficiency	4 (0.6%)	78 (48 – 144)	Hypo-IgA and hypo-IgM Reduced switched memory B cells Elevated transitional B cells CD4 lymphopenia	100% 100% 100% 100%
	LRBA deficiency	3 (0.4%)	216 (156 – 336)	Hypogammaglobulinemia Reduced switched memory B cells Expanded CD21^{lo} B cells	100% 100% 100%
	TPP2 deficiency	2 (0.3%)	106 (32 – 180)	Hypogammaglobulinemia Reduced naïve T cells	100% 100%
	IPEX syndrome	1 (0.1%)	17	Markedly decreased FoxP3^{+}CD25^{+} regulatory T cells	100%
Congenital defects of phagocyte number or function		20 (3.0%)	23 (1 – 372)		
	Congenital neutropenia	8 (1.2%)	22.5 (3 – 36)		
	Cyclic neutropenia	2 (0.3%)	26.5 (23 – 30)		
	CGD	7 (1.0%)	36 (5 – 372)	Absent/markedly reduced NADPH oxidase activity	100%
	LAD-1	2 (0.3%)	9.5 (1 – 18)	Absent CD18/CD11a expression	100%
Defects in intrinsic and innate immunity		12 (1.8%)	66 (18 – 252)		
	MSMD	4 (0.6%)	78 (30 – 252)		
	CMC	5 (0.7%)	36 (24 – 72)	IL-17A production defect Hyperphosphorylation of STAT1 after IFN-γ stimulation	100% 100%
Autoinflammatory disorders		2 (0.3%)	84 (72 – 96)		
	Blau syndrome	1 (0.1%)	72		
	A20 deficiency	1 (0.1%)	96		
Complement deficiencies		153 (22.8%)	288 (12 – 972)		
	C1 inhibitor deficiency	115 (17.2%)	336 (12 – 972)		
	Factor I deficiency	5 (0.7%)	426 (252 – 552)		
	Factor H deficiency	3 (0.4%)	348 (12 – 468)		
	CD46 deficiency	7 (1%)	240 (144 – 456)	Absent CD46 expression on lymphocytes Reduced CD46 expression on lymphocytes	85.7% 14.3%
	C3 deficiency	2 (0.3%)	96 (72 – 120)		

(Continued)

TABLE 2 Continued

Category	Disease	N (%)	Age at diagnosis (months) median (range)	Associated flow cytometry findings	
				Findings	*%*
	C7 deficiency	1 (0.1%)	264		
	CHAPLE disease	6 (0.9%)	72 (24 – 168)	Absent CD55 expression on neutrophils	100%
Phenocopies of IEI	Good syndrome	3 (0.4%)	780 (636 – 828)	Hypogammaglobulinemia Profoundly decreased B cells Inverted CD4/CD8 ratio	100% 100% 100%
Unclassified immunodeficiencies		12 (1.8%)	36 (7 – 516)		

*: naïve and memory T cells were analyzed in 72 patients.
**: WASp expression was assessed in 9 patients.
#: Th17, Il-17 production and STAT3 phosphorylation were assessed in 22 patients.
¥: Btk expression was assessed in 2 male patients.
§: Phenotyping of circulating B-cell subpopulations was performed in 63 patients.
£: NK-cell degranulation assay was performed in two patients.
ALPS, autoimmune lymphoproliferative syndrome; APDS, activated PI3K delta syndrome; ARPC1B, actin related protein 2/3 complex subunit 1B; Btk, Bruton's tyrosine kinase; CGD, chronic granulomatous disease; CID, combined immunodeficiency; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CMC, chronic mucocutaneous candidiasis; CVID, common variable immunodeficiency; FHL, familial hemophagocytic lymphohistiocytosis; HLA, human leukocyte antigen; IPEX, immune dysregulation-polyendocrinopathy-enteropathy-x-linked; LRBA, LPS-responsive beige-like anchor protein; MHC, major histocompatibility complex; MSMD, mendelian susceptibility to mycobacterial disease, OS, Omenn syndrome; RIPK1, receptor-interacting serine/threonine-protein kinase 1; RTE, recent thymic emigrants; SCID, severe combined immunodeficiency; SIAD, selective IgA deficiency; sIg, serum immunoglobulins; TPP2, tripeptidyl peptidase 2; STAT, signal transducer and activator of transcription; WASp, Wiskott Aldrich syndrome protein.

TABLE 3 IEI causing genes identified in our series.

Category	Disease	Inheritance	Gene	Number of patients
Combined immunodeficiencies	T^-B^+ SCID	XL	*IL2RG*	1
	T^-B^- SCID	AR	*RAG1*	2
		AR	*RAG2*	1
		AR	*ADA*	1
	Omenn Syndrome	AR	*RAG1*	4
		AR	*RAG2*	1
	$T^{low}B^{-/low}$ leaky SCID	AR	*RAG1*	2
		AR	*RAG2*	2
		AR	*DCLRE1C*	1
		AR	*LIG4*	1
		AR	*ADA*	1
	$T^{low}B^+$ leaky SCID	AR	*CORO1A*	2
	MHC-II deficiency	AR	*RFXANK*	10
	MHC-I deficiency	AR	*TAP2*	1
	HIMS	AR	*CD40*	2
	CD3γ deficiency	AR	*CD3G*	2
	FCHO1 deficiency	AR	*FCHO1*	2
		AR	*DOCK8*	1
	DOCK8 deficiency			
	SASH3 deficiency	XL	*SASH3*	1
	IKBKB deficiency	AR	*IKBKB*	1
	HELIOS deficiency	AR	*IKZF2*	1
CIDs with associated or syndromic features	Wiskott-Aldrich syndrome	XL	*WAS*	2
	Arp2/3-mediated filament branching defect	AR	*ARPC1B*	2
	AD-HIES STAT3 deficiency (Job syndrome)	AD	*STAT3*	1
	Vici syndrome	AR	*EPG5*	1
Predominantly antibody deficiencies	XLA	XL	*BTK*	7
	Activated p110δ syndrome	AD	*PIK3CD GOF*	2
	TACI deficiency	AD	*TNFRSF13B*	1

(Continued)

TABLE 3 Continued

Category	Disease	Inheritance	Gene	Number of patients
Diseases of immune dysregulation	Chediak-Higashi Syndrome	AR	*LYST*	7
	Munc13–4 deficiency (FHL3)	AR	*UNC13D*	1
	LRBA deficiency	AR	*LRBA*	3
	RIPK1 deficiency	AR	*RIPK1*	4
	Tripep tidyl-Peptidase II Deficiency	AR	*TPP2*	2
	ALPS-FAS	AR	*TNFRSF6*	2
Defects in intrinsic and innate immunity	MSMD	AR	*IL12RB1*	2
		AR	*TYK2*	1
	CMC	AD	*STAT1 GOF*	2
Autoinflammatory disorders	Blau syndrome	AD	*NOD2*	1
	A20 deficiency	AD	*TNFAIP3*	1
Complement deficiencies	C3 deficiency	AR	*C3*	2
	CD46 deficiency	AR	*CD46*	3
	CHAPLE disease	AR	*CD55*	6

ALPS, autoimmune lymphoproliferative syndrome; ARPC1B, actin related protein 2/3 complex subunit 1B; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CMC, chronic mucocutaneous candidiasis; FCHO1, F-BAR domain only protein 1; FHL, familial hemophagocytic lymphohistiocytosis; HIMS, hyper-IgM syndrome; HIES, hyper-IgE syndrome; IKBKB, inhibitor of nuclear factor kappa-B kinase, subunit beta; LRBA, LPS-responsive beige-like anchor protein; MHC, major histocompatibility complex; MSMD, mendelian susceptibility to mycobacterial disease; RIPK1, receptor-interacting serine/threonine-protein kinase 1; SCID, severe combined immunodeficiency; TACI, transmembrane activator and CAML interactor; WAS, Wiskott Aldrich syndrome; XLA, X-linked agammaglobulinemia.

3.2.2 Omenn syndrome and leaky SCIDs

Eight patients (4 males and 4 females) were diagnosed with OS. The median age at diagnosis was 4.5 months. All patients exhibited $T^{low/norm}$B-NK+ profile with T-cell count ranging from 340/µl to 18360/µl (median=533/µl). Circulating B cells were absent in all patients, while NK-cell count ranged from 298 to 2123/µl (median=715/µl). The T-cell subpopulation analysis was notable for the absence of naïve T cells and RTE in all patients (Table 2). Genetic analysis was performed in 5 patients revealing homozygous mutations of *RAG1* in four patients and a homozygous variant of *RAG2* in another one (Table 3).

Nine patients (4 males and 5 females) carried homozygous hypomorphic variants in *RAG1* (n=2), *RAG2* (n=2), *DCLRE1C* (n=1), *LIG4* (n=1), *ADA* (n=1), and *CORO1A* (n=2), and presented with leaky SCID (i.e., 7 patients with T^{low} $B^{-/low}$ leaky SCID and 2 patients with T^{low} B^{+} leaky SCID). The median age at diagnosis was 19 months (range: 5 – 240) and the median age of symptoms onset was 6 months (range: 4 – 60). The median T-cell count was 614/µl (range: 471 – 2530) and the percentage of CD4+CD45RA+ and CD8+CD45RA+CCR7+ T cells ranged from 0.7% to 25.0% (median=6.5%) and 0.0% to 19.0% (median=6.3%), respectively. RTE were very low in all patients with a median of 1.7% (range 0% – 7.5%). Among patients with T^{low} $B^{-/low}$ leaky SCID, B-cell count was decreased or markedly decreased in all patients, with a median of 24/µl (range: 4 – 261) (Figure 2).

3.2.3 Bona fide CIDs

One hundred sixteen patients were diagnosed with CID based on FCM analysis. CD3 lymphopenia was seen in 61 (52.6%) patients, CD4 and CD8 lymphopenia were present in 91 (78.4%) and 43 (37.1%) patients respectively, while inverted CD4/CD8 ratio was noted in 75 (64.7%) patients. Extended analysis of T-cell subpopulations was performed in 90 (77.6%) patients, revealing expanded γδ T cells in 8 (8.9%) patients, and reduced naïve CD4 and CD8 T cells in 76 (84.4%) and 79 (87.8%) patients, respectively. Genetic testing was performed in 35 patients revealing 9 different disorders, i.e., RFXANK, CD40, DOCK8, CD3γ, FCHO1, SASH3, IKBKB, HELIOS, and TAP2 deficiencies, in 21 patients (Table 3). CD3γ deficiency was diagnosed in two siblings presenting with recurrent infections (recurrent pneumonia, multiple abscesses and candidiasis), lymphoproliferation and autoimmune hemolytic anemia. Interestingly, FCM analysis was notable for quantitative variations of circulating T and B cells (T-cell lymphopenia, markedly decreased naïve T cells, and expanded $CD21^{lo}$ B cells), but also for low expression of the TCR-CD3 complex on the T-cell surface.

MHC-II deficiency was the most frequent CID in our series with 35 cases, representing 21.5% of the total number of CIDs. Typically, MHC-II deficiency is characterized by hypogammaglobulinemia, CD4 lymphopenia and absent HLA-II expression on B cells and monocytes. In our series, low CD4 T-cell count was seen in 31 (88.6%) patients, inverted CD4/CD8 ratio in 26 (74.3%) patients, and CD8 lymphopenia in 10 (28.6%) patients. Decreased IgG, IgA and/or IgM were noted in 19 (54.3%), 26 (74.3%), and 15 (42.9%) patients, respectively. HLA-DR expression at the surface of B cells was absent in 34 patients and markedly decreased in one patient.

3.3 CIDs associated with syndromic features

3.3.1 Wiskott-Aldrich syndrome

Sixteen patients were diagnosed with WAS in our center. Thirteen patients (81.2%) showed decrease in CD4 T cells, CD8 T cells or both. CD4/CD8 ratio was low in 10 patients (62.5%) and

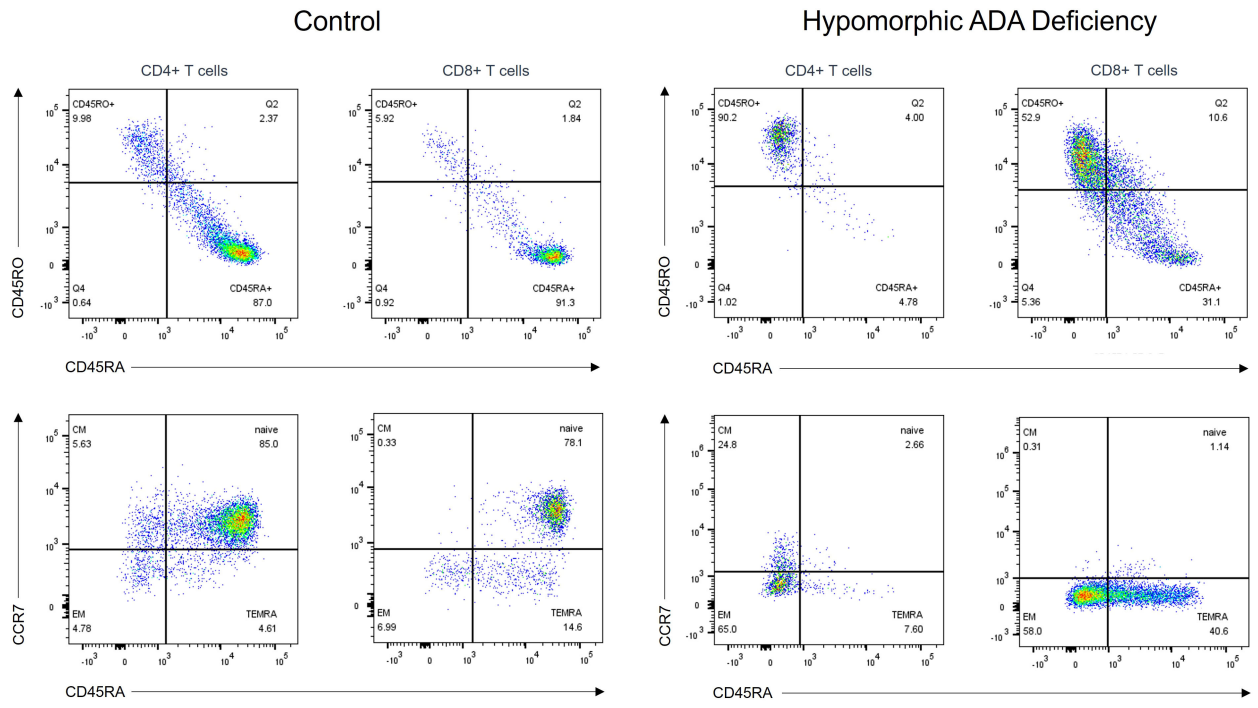

FIGURE 2
T-cell subpopulation analysis in a patient with hypomorphic ADA deficiency and an age-matched healthy control. T cells from the patient, carrying a homozygous ADA variant (c.965T>C= p.Phe322Ser), exhibit a memory phenotype (CD45RO+) with nearly absent native (CD45RA+CCR7+) subsets. ADA, adenosine deaminase.

high in 4 (25%). Intracytoplasmic staining for WASp was performed in 9 patients unveiling reduced or absent WASp expression in six patients.

3.3.2 Hyper-IgE syndrome

Fifty-seven patients had hyper-IgE (i.e., IgE > 10 times the norm for age) and pathologic susceptibility to infections with no evidence of T-cell or B-cell deficiency. They were diagnosed with HIES according to ESID criteria. Th17 cells, IL-17 production and STAT3 phosphorylation were assessed in 22 patients. Defective IL-17 production was found in 18 (81.8%) patients, low Th17 cells in 10 (45.5%) patients, while a reduced or absent STAT3 phosphorylation assay was observed in two (9.1%) patients (Table 2, Figure 3).

3.4 Predominantly antibody deficiencies

One hundred fifty-five patients were diagnosed with PAD. Among them, 17 (11.0%) had Agammaglobulinemia, 70 (45.2%) had common variable immunodeficiency (CVID), and 15 (9.7%) had selective IgA deficiency (Table 2).

3.4.1 Agammaglobulinemia

Seventeen patients, including 13 males and 4 females, were diagnosed with Agammaglobulinemia. Sequencing of the *BTK* gene was performed in ten male patients. Seven of them (70%) had hemizygous pathogenic variants in *BTK*. Intracytoplasmic Btk staining was carried out in two patients, both of them displayed absent Btk expression.

3.4.2 CVID

Seventy patients were diagnosed with CVID based on a comprehensive assessment of their clinical manifestations, immunoglobulin levels, and FCM analysis of B- and T-cell subsets. B-cell subpopulation analysis was performed in 63 patients demonstrating a significant reduction in switched memory B cells in 51 (80.9%) patients. Additionally, an expansion of transitional B cells and $CD21^{lo}$ B cells was seen in 23.8% and 28.6% of patients, respectively (Figure 4). Genetic analysis through whole exome sequencing (WES) was carried out in three patients, revealing two cases of activated phosphoinositide 3 kinase (PI3K)-δ syndrome (APDS) caused by heterozygous GOF mutations in *PIK3CD*, and one TACI deficiency due to heterozygous mutation in *TNFRSF13B*. Both patients with APDS exhibited decreased naïve T cells, reduced number of switched memory B cells, and very high percentage of transitional B cells (Table 2).

3.5 Diseases of immune dysregulation

Forty-nine patients were diagnosed with a disorder of immune dysregulation. Among them, nine had CHS, seven had FHL, five had ALPS, two had autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and one had IPEX syndrome.

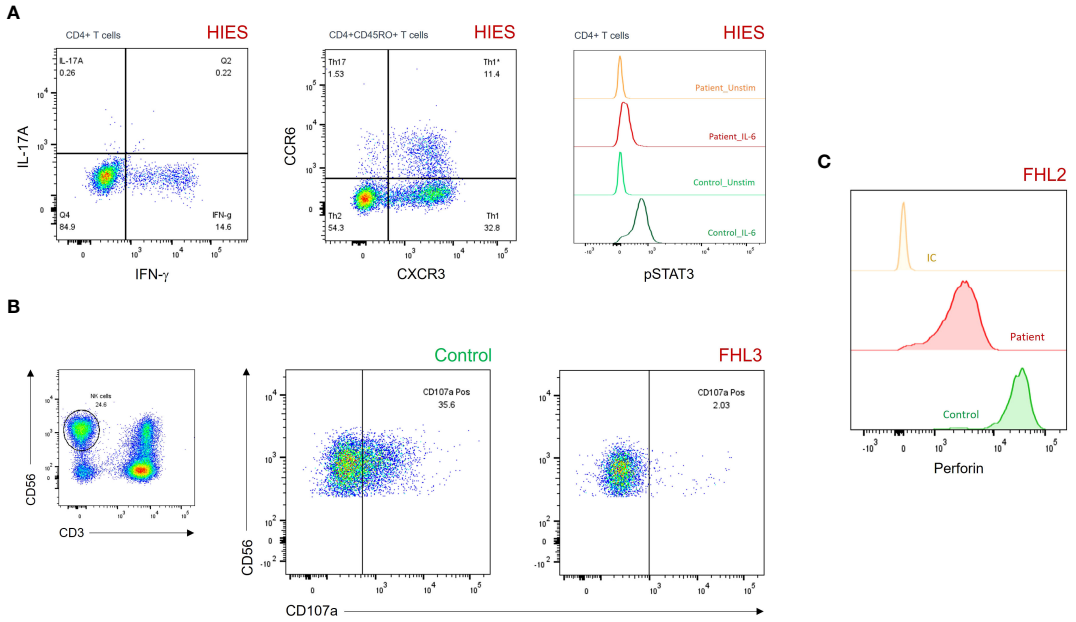

FIGURE 3
(A) Dot plots and histograms showing reduced IL-17A production (upper left panel), CD45RO$^+$CCR6$^+$CXCR3$^-$ Th17 cells (upper middle panel), and pSTAT3 levels after IL-6 stimulation (upper right panel) in a patient with HIES. **(B)** Degranulation assay of resting NK cells showing impaired CD107a surface expression in a patient with FHL3. **(C)** Histograms showing partial perforin expression defect on gated CD3$^-$CD56$^+$ NK cells in a patient with FHL2. FHL, familial hemophagocytic lymphohistiocytosis; HIES, hyper-IgE syndrome; IC, isotypic control; pSTAT3, phosphorylated signal transducer and activator of transcription 3.

Twenty patients (40.8%) received genetic testing. Biallelic germline pathogenic variants were identified in six different genes, i.e., *LYST*, *FAS*, *LRBA*, *UNC13D*, *RIPK1*, and *TPP2* (Table 3).

Elevated CD3+TCRαβ+CD4−CD8−T cells (>2.5% of CD3+ lymphocytes) was found in five unrelated patients with ALPS; two of them carried the same missense homozygous mutation (p. Phe133Val) in *FAS*. Increased TCRαβ DNT were also found in nine patients with other immunodeficiency disorders such as: CVID, STAT1-GOF, LRBA, ARPC1B, RIPK1, and SASH3 deficiencies. However, the expansion of TCRαβ DNT was more pronounced in patients with ALPS (ALPS: median= 22%, range: 8% – 67%; Other IEIs: median= 3.5%, range: 3% – 23%).

LRBA deficiency was diagnosed in three patients, all of whom presented with typical features, including autoimmune cytopenia, granulomatous-lymphocytic interstitial lung disease (GLILD), hypogammaglobulinemia, reduced switched memory B cells, and expanded CD21^{lo} B cells.

One patient out of the seven diagnosed with FHL exhibited a decreased perforin expression (as evidenced by a 10-fold reduction in MFI in comparison to control), despite maintaining a normal percentage of positive NK cells (97% of positivity) (Figure 3). Of the six remaining patients, five displayed defective/abnormal NK-cell degranulation. Moreover, two of three patients (85%) with CHS or GS2 had defective NK-cell degranulation (Figure 3).

All patients with RIPK1 deficiency presented with early-onset inflammatory bowel disease and decreased serum IgA and IgM levels. The FCM analysis unveiled reduced numbers of memory B cells and a concurrent elevation of transitional B cells in all cases.

3.6 Defects of phagocytes

Seven out of the 20 patients with phagocytic defects were diagnosed with CGD based on the FCM measurement of NADPH oxidase function. Notably, three male patients demonstrated a complete absence of respiratory burst, while four additional patients (2 males and 2 females) exhibited markedly reduced NADPH oxidase activity, indicated by a stimulation index (SI) ranging from 6 to 54 (Table 2).

3.7 Innate immunity defects

Four patients were diagnosed with MSMD; two of them had IL-12Rβ1 deficiency confirmed genetically. On patient had compound heterozygous mutations in *TYK2*, while the WES results weren't conclusive for the last patient. Five patients with CMC displayed IL-17A production defect and hyperphosphorylation of STAT1 after IFN-γ stimulation. Heterozygote pathogenic variant in *STAT1* was identified in two patients (Tables 2, 3).

3.8 Complement deficiencies

Patients with suspected aHUS were systematically screened for CD46 deficiency. Complete lack of CD46 expression (i.e., homozygous CD46 deficiency) was found in six patients, while partial defect of CD46 expression (likely heterozygous CD46

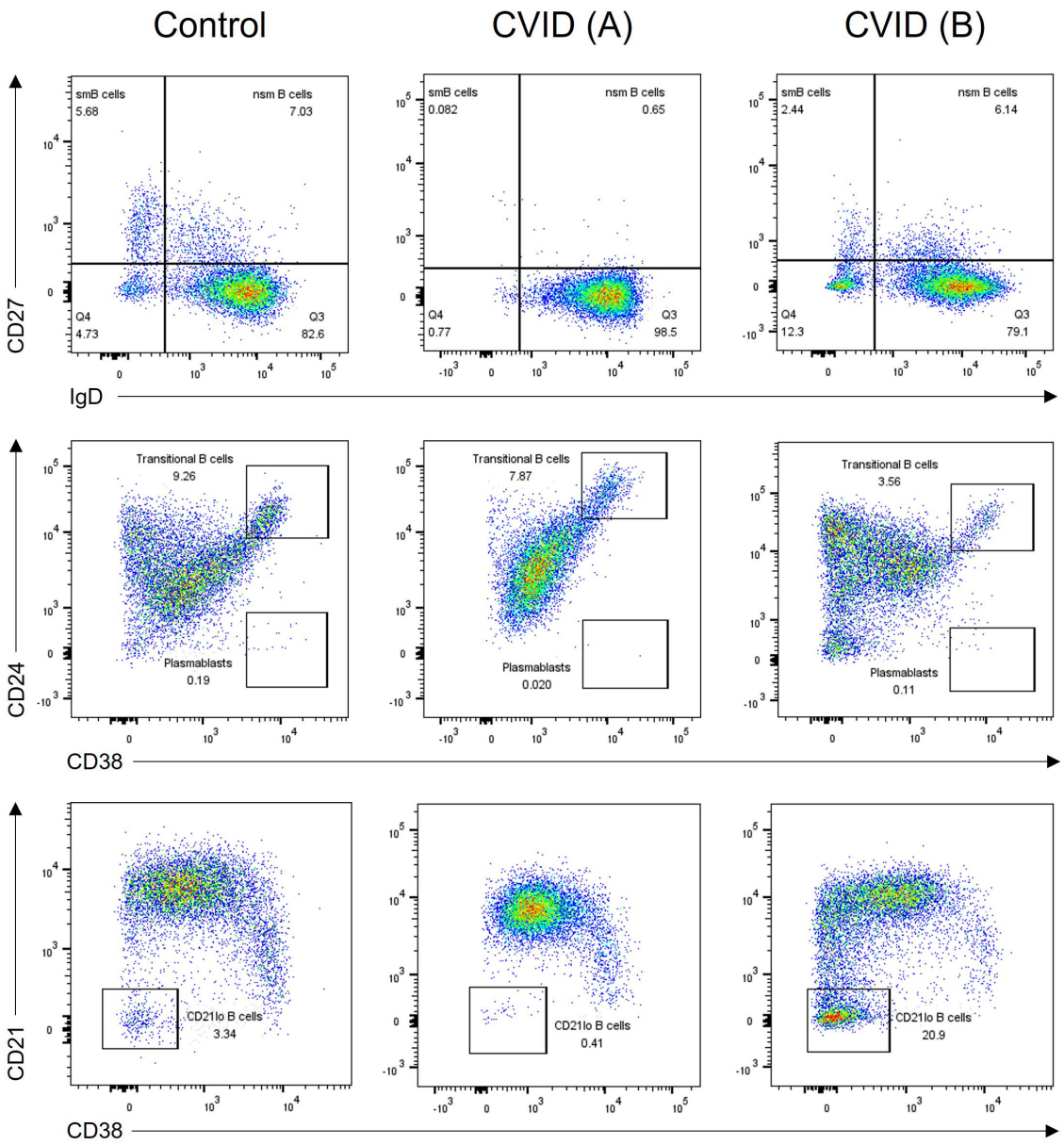

FIGURE 4
B-cell subpopulation analysis in two CVID patients and one healthy control. *CVID patient (A)* has reduced memory B-cell subsets including switched memory (CD27+sIgD-) and non-switched memory (CD27+sIgD+) B cells (middle panel). *CVID patient (B)* shows an expansion of $CD21^{lo}$ B cells (right panel). CVID, common variable immunodeficiency; sm, switched memory; nsm, non-switched memory.

deficiency) was seen in one patient. Parents of patients with homozygous CD46 deficiency exhibited a heterozygous profile with a CD46 expression density representing ~ 50% of that of healthy controls. Based of FCM analysis, the diagnosis of homozygous CD55 (DAF) deficiency was established in six patients presenting with complement hyperactivation, angiopathic thrombosis, and protein-losing enteropathy (CHAPLE) disease. The assessment of CD55 expression in the family members of three patients unveiled an intermediate expression pattern, indicating a partial (heterozygous) defect in CD55 expression among the carrier parents (Figure 5).

4 Discussion

The department of medical biology at Rouiba hospital is one of the few centers in Algeria offering a comprehensive workup for patients with suspected IEI. In this study, we conducted a retrospective review of the use of FCM in the diagnosis of IEIs. Sharing insights into our practical experiences, we present FCM-based diagnostic approaches adapted to different clinical scenarios. In the past two decades, FCM has emerged as a highly valuable and versatile tool for diagnosing and studying IEIs (12). Individual FCM diagnostic testing depends on the patient's clinical presentation and basic laboratory findings. The range

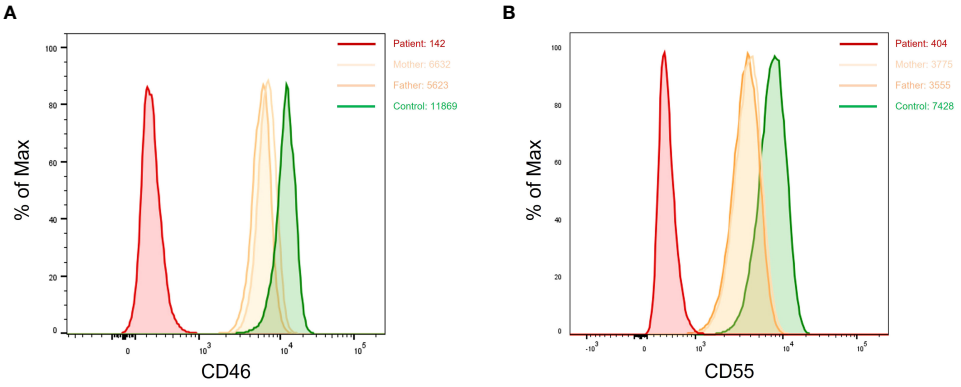

FIGURE 5
Dot plots showing complete loss of CD46 **(A)** and CD55 **(B)** expression in two patients with aHUS and CHAPLE disease, respectively. Carrier parents from both families show monomodal and intermediate expression of CD46 and CD55. aHUS, atypical hemolytic uremic syndrome; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy.

of applications is broad, including phenotypic assays that investigate the numbers and percentages of immune cells (e.g., RTE, TCRαβ DNT, switched memory B cells), functional analysis of cellular processes (e.g., cytokine secretion, STAT phosphorylation, NK cell degranulation, NADPH oxidase activity in neutrophils), and direct analysis of potentially mutated cell membrane, and cytoplasmic proteins (Table 1) (9, 12, 19). The use of this diverse array of phenotypic and functional assays has proven instrumental in establishing definitive diagnoses for various disorders. However, it is important to note that these assays, if not conducted with meticulous attention to preanalytical and analytical considerations, may produce inaccurate results, potentially leading to misdiagnosis (12, 13, 20, 21). To implement such diagnostic assays with high and reproducible quality, a high level of expertise is required. Specifically, for protein expression and functional assays, in-house reference values need to be determined, and the parallel testing of healthy controls is highly recommended (9).

Over a nearly seven-year period, our laboratory diagnosed a total of 670 patients, with 70 different IEIs categorized into 9 different groups according to the IUIS classification (Tables 2, 3). FCM has proven useful in diagnosing and categorizing IEI in 514 patients (76.7%). Based on our experience, FCM provided direct diagnostic insights for immunodeficiencies such as SCID, OS, MHC-II deficiency, WAS, XLA, XL-CGD, FHL, LAD-1, AR-CD46 deficiency, and CD55 deficiency. For certain IEIs, including HIES, STAT1-GOF, APDS, ALPS, IPEX, AR-CGD, and autosomal dominant (AD)-CD46 deficiency, FCM offered suggestive evidence, necessitating subsequent genetic testing for confirmation. FCM findings provided informative clues, although they lacked specificity for diagnosing hypomorphic V(D)J recombination defects, Ataxia Telangiectasia, CVID, LRBA deficiency, RIPK1 deficiency, and Good's syndrome (Table 4). Additionally, in select IEIs such as selective IgA deficiency, transient hypogammaglobulinemia of infancy, congenital neutropenia, and early-onset inflammatory bowel disease, FCM played a crucial role in differential diagnosis and narrowing down possibilities.

In our laboratory, the flow cytometric analysis is systematically structured according to the clinical scenario (Figure 6). For patients presenting with suspected CID, characterized by early onset severe/recurrent bacterial, fungal, and viral infections, along with severe reactions to live microorganism vaccines, the flow cytometric work-up starts with basic lymphocyte phenotyping. The identification of profound T lymphopenia (i.e., T cell count <300/µl) serves as a direct clue for the diagnosis of SCID (22). Simultaneous analysis of B and NK cell subpopulations aids in classifying SCID and facilitates the optimization of genetic testing through targeted Sanger sequencing. In our series, 30 out of the 163 patients (18.4%) diagnosed with combined immunodeficiency had SCID with a notable 70% exhibiting T-B-NK+ SCID. In contrast to patients with typical SCID, those harboring hypomorphic variants in SCID-causing genes typically manifest a less pronounced lymphopenia, thereby necessitating a more detailed analysis of naïve and memory subpopulations. Interestingly, despite substantial number of circulating T cells, naive T cells were absent in all patients with OS diagnosed in our laboratory (Table 2). Delving into the analysis of naive T cells, and more interestingly RTE in these patients, provides compelling evidence for an accurate diagnosis (Figure 6) (23). In addition to OS, leaky SCIDs form another category within the hypomorphic SCID spectrum. Patients with leaky SCID most often present with atypical phenotype characterized by less severe-but atypical-infections with a later onset, and a high frequency of immune dysregulation features, particularly autoimmune cytopenia (2, 24–26). T-B-NK enumeration in these patients often reveals slightly decreased to normal T-cell count, leading to a misdiagnosis (24). We previously reported a case of hypomorphic Artemis deficiency in a young adult, characterized by recurrent pneumonia, epidermodysplasia verruciformis, and a subtle T cell lymphopenia coupled with severe decrease in RTE (27). Given the diagnostic challenges inherent to such atypical entities, it appears reasonable to incorporate detailed T-cell phenotyping as a first-line investigation, optimizing diagnostic accuracy and minimizing the potential for misdiagnosis.

One hundred sixteen patients were diagnosed with *bona fide* CID. The most common immunophenotypic features were CD4 lymphopenia (78.4%), inverted CD4/CD8 ratio (64.7%), and

TABLE 4 Flow cytometry-based diagnosis of IEIs.

IEI	Associated flow cytometry findings
Robust evidence	
SCID	T-cell count <300/µl
Omenn Syndrome	Absent or severely decreased naïve T cells and RTE
MHC-II deficiency	Absent or markedly decreased HLA-DR expression on B cells
WAS	Absent or markedly decreased WASp expression on T cells
XLA	Absent or very low (<2%) circulation B cells with absent/reduced Btk expression on monocytes
XL-CGD	Absent NADPH oxidase activity
FHL type 2	Absent or markedly decreased perforin expression on NK cells
FHL types 3, 4 and 5	Defective resting NK-cell degranulation assay
LAD-1	Absent CD18/CD11a expression on neutrophiles
AR-CD46 deficiency	Absent CD46 expression on T cells
CHAPLE disease	Absent CD55 expression on neutrophiles
Suggestive evidence	
CD3γ deficiency	Low CD3/TCRαβ expression on T cells
HIES	Markedly decreased Th17 cells or defective IL-17 production associated or not with STAT3 phosphorylation defect
CMC-STAT1 GOF	Markedly decreased Th17 cells or defective IL-17 production associated with hyperphosphorylation of STAT1
APDS	Expanded transitional B cells with reduced switched memory B cells and decreased naïve T cells
ALPS-FAS	Significant increase in TCRαβ DNT (>7% of CD3 + T cells)
IPEX syndrome	Absent or markedly decreased $FOXP3^{+}CD25^{+}$ Treg
AR-CGD	Reduced NADPH oxidase activity
AD-CD46 deficiency	~ 50% reduction of CD46 expression on lymphocytes
Informative clues	
Hypomorphic V(D)J recombination defects	Markedly decreased naïve T cells and RTE associated or not with reduced B cells
DiGeorge syndrome	Reduced naive T cells and RTE
Ataxia Telangiectasia	CD3 lymphopenia with reduced naive T cells
CVID	Markedly decreased switched memory B cells
LRBA deficiency	Reduced switched memory B cells with expanded $CD21^{lo}$ B cells
RIPK1 deficiency	Decreased central and effector memory CD4 T cells Decreased switched and non-switched memory B cells Raised transitional B cells
Good's syndrome	Absent or markedly decreased B cells Low CD4/CD8 ratio

AD, autosomal dominant; ALPS, autoimmune lymphoproliferative syndrome; APDS, activated PI3K delta syndrome; AR, autosomal recessive; CGD, chronic granulomatous disease; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CMC, chronic mucocutaneous candidiasis; CVID, common variable immunodeficiency; FHL, familial hemophagocytic lymphohistiocytosis; HIES, hyper-IgE syndrome; IPEX, immune dysregulation-polyendocrinopathy-enteropathy-x-linked; LRBA, LPS-responsive beige-like anchor protein; LAD, leukocyte adhesion deficiency; LRBA, LPS-responsive beige-like anchor protein; MHC, major histocompatibility complex; RIPK1, receptor-interacting serine/threonine-protein kinase 1; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; WAS, Wiskott Aldrich syndrome; XLA, X-linked agammaglobulinemia.

reduced naïve CD4 (84.4%) and CD8 (87.8%) T cells. In addition to quantitative abnormalities, lymphocyte phenotyping can unveil subtle aberrations, such as low expression of CD3 complex on the T-cell surface. Indeed, we observed in two siblings, both affected with CD3γ deficiency, a 10-fold lower expression of the TCR-CD3 complex on the surface of T cells. Both brothers exhibited a clinical picture of CID with recurrent infections, autoimmune cytopenia, and lymphopenia. In view of these findings, FCM analysis should also carefully consider the intensity of CD3 expression.

MHC class II deficiency was the most prevalent CID in our series, accounting for 21.5% of all CIDs and 5.2% of total IEI cases. Genetic analysis conducted in 12 patients revealed pathogenic variants in the *RFXANK* gene in 10 patients, with 9 displaying an identical 26-base pair deletion (752delG26). The high frequency of the 752delG26 deletion in our patients is an accordance with earlier findings, underscoring a high occurrence of the same variant in over 90% of North African families (28, 29). This finding appears to be associated with a founder effect related to this mutation, as previously suggested by Ouederni et al. (30). Typically, the suspicion of MHC-II deficiency is raised in patients with recurrent infections, hypogammaglobulinemia, CD4 lymphopenia, and low CD4/CD8 ratio (31). However, it is noteworthy to mention that patients with MHC-II deficiency may paradoxically manifest a normal CD4+ T-cell count and serum immunoglobulin (Ig) levels. Notably, in our series, 11.4% of MHC-II deficient patients displayed a normal CD4+ T-cell count, 25.7% had normal CD4/CD8 ratio, and 45.7% maintained IgG concentration within the normal range (Table 2). Hence, it would be relevant to systematically screen patients of North African descent exhibiting a clinical presentation of CID for MHC-II deficiency, particularly in cases involving parental consanguinity, recurrent pneumonia, and chronic diarrhea.

Hyper IgE syndrome is a heterogeneous group of monogenic disorders characterized by the triad of high serum IgE levels, eczema, and recurrent skin and lung infections. AD-HIES caused by LOF mutations in *STAT3* gene is the prototype of these disorders. Additionally, six recently identified disorders (IL6ST, IL6R, ZNF341, ERBIN, CARD11, and TGFBR deficiencies) were

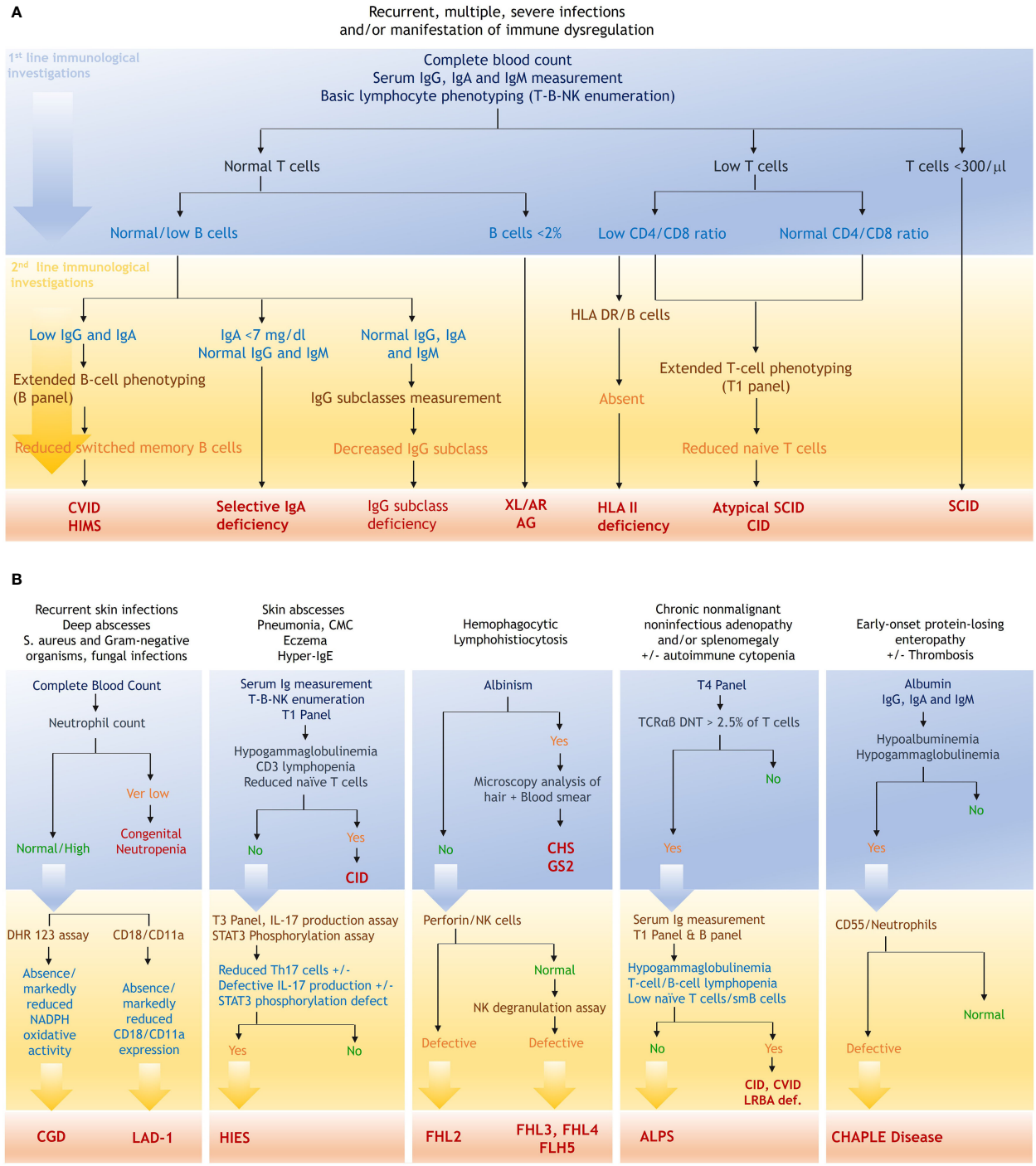

FIGURE 6
Flow cytometric workup based on the clinical presentation. **(A)**, FCM based strategy in the context of nonspecific IEI manifestations. **(B)**, diagnostic strategy in the context of specific clinical presentations. *T1 panel, CD3, CD4, CD8, CD45RA, CD45RO, CCR7; T3 panel, CD3, CD4, CD45RO, CXCR3, CCR6, CXCR5; T4 panel, TCRαβ, TCRγδ, CD3, CD4, CD8, CD45; B panel, CD19, CD27, sIgD, CD38, CD24, CD21*. ALPS, autoimmune lymphoproliferative syndrome; AR, autosomal recessive; CGD, chronic granulomatous disease; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CHS, Chediak-Higashi syndrome; CID, combined immunodeficiency; CVID, common variable immunodeficiency; DHR, Dihydrorhodamine; GS2, Griscelli syndrome type 2; HIES, hyper-IgE syndrome; HIMS, hyper-IgM syndrome; LAD, leukocyte adhesion deficiency; LRBA, LPS-responsive beige-like anchor protein; SCID, severe combined immunodeficiency; STAT, signal transducer and activator of transcription; XLA, X-linked agammaglobulinemia.

included in the HIES spectrum, along with PGM3 and SPINK5 deficiencies (1, 32). Interestingly, high total IgE levels have also been reported in association with some CIDs, including DOCK8 deficiency, WAS, and ARPC1B deficiency (33–36). Accordantly, a crucial initial step in our approach for patients with suspected HIES is to rule out the presence of CID through a comprehensive T-cell phenotyping. Low Th17 cells and defective IL-17 production are characteristic features of patients with STAT3 deficiency and

STAT3–related disorders, such as gp130, IL-6R, ZNF341, and PGM3 deficiencies (37–43). The analysis of CXCR3 and CCR6 markers on CD4+ memory T cells provides a straightforward approach for investigating Th17 lymphocytes (18). However, based on our experience, this phenotypic assay seems to be less sensitive in detecting impaired Th17 immunity compared to IL-17 production assay (Table 2). Furthermore, patients with certain disorders sharing clinical features with HIES, such as IL-21R deficiency, may exhibit diminished Th17 cells (44). The STAT3 phosphorylation assay, originally designed for STAT3 deficiency, does not consistently exhibit abnormalities in cases of STAT3 deficiency depending on the mutation's location (45). Moreover, other STAT3-related HIES frequently manifest a defective STAT3 phosphorylation (39–43).

In the context of suspected primary antibody production defect, the flow cytometric work-up usually starts with T-B-NK enumeration (Figure 6). The absence or extremely low levels of B cells (<2% of circulating lymphocytes), accompanied by a significant reduction in total serum Ig levels and normal T cells strongly indicate a diagnosis of Agammaglobulinemia. Male patients with Agammaglobulinemia should be systematically screened for XLA-Btk deficiency. Since the introduction of the Btk expression assay in our laboratory, two patients have been tested and exhibited markedly decreased Btk expression in monocytes. According to the literature data, the majority of identified Btk mutations impair or abrogate Btk protein expression (9, 46). However, normal Btk protein levels do not exclude XLA, and in cases where clinical suspicion is high, genetic analysis should be performed (9, 15). Beyond the scope of Agammaglobulinemia, most patients with PAD display a less severe B lymphopenia, necessitating further B cell subset analysis (Table 1, Figure 6). Disturbances in B-cell subpopulations, such as reduced memory B cells as well as elevated transitional or $CD21^{lo}$ B cells, provide direct insights into a primary origin for the antibody production defect, especially in adult patients where secondary causes of hypogammaglobulinemia are common (47).

Common variable immunodeficiency was the most prevalent PAD in our series, accounting for 45.2% of all PADs and 10.4% of total IEI cases. CVID is a heterogenous group of monogenic disorders characterized by reduced serum levels of IgG, IgA, and/or IgM, with impaired antibody response to both polysaccharide and protein antigens. Clinically, CVID patients suffer from an increased susceptibility to sinopulmonary infections along with a broad spectrum of inflammatory, granulomatous, and autoimmune diseases (48–50). Reduced numbers of switched memory B-cells, found in 80.9% of our CVID patients, represent the cellular hallmark of CVID and serve as a key diagnostic element according to ESID criteria (51, 52). Additional B-cell disturbances like expansion of transitional or $CD21^{lo}$ B cells were also found in our CVID cohort. Genetic analysis through WES identified two patients with heterozygous GOF mutations in *PIK3CD*. Both patients presented with APDS symptoms (i.e., sinopulmonary infections, lymphoproliferation, enteropathy, and autoimmune hemolytic anemia), and displayed a characteristic immune profile including increased proportion of transitional B cells, reduced memory B cells, decreased naïve T cells, and elevated levels of serum IgM (53, 54).

A significant number of patients with CVID present some features of a cellular immunodeficiency and may, in fact, suffer from a certain form of CID (55). To exclude severe T-cell deficiency in patients with suspected CVID, we systematically conducted a comprehensive analysis of naïve and memory T-cell subpopulations. The revised criteria from the ESID registry require therefore a T-cell count of >200/µl with an amount of at least 10% of naïve CD4+CD45RA+ T cells in adults (52). von Spee-Mayer et al, reported that a reduction in naïve CD4 T cells to less than 10% had the highest sensitivity of all tested markers for patients with clinical complications often associated with CID, although the authors admitted that none of the current definitions sufficiently separates CID from CVID patients (56). It is noteworthy that both patients with APDS, as well as the one with TACI deficiency, exhibited a significant reduction in naïve CD4 T cells (less than 5%), despite being categorized as CVID.

Forty-nine patients were diagnosed with immune dysregulation disorders, including seven with FHL, five with ALPS, and three with LRBA deficiency. Elevated TCRαβ DNT represent a distinctive feature of ALPS and serve as a major diagnostic criterion (57). However, according to our experience, elevated TCRαβ DNT may also be found in other monogenic disorders, such as LRBA, ARPC1B, RIPK1, and SASH3 deficiencies, as well as CVID, and CMC with STAT1-GOF. Therefore, in addition to the clinical presentation, the extent of the expansion of TCRαβ DNT cells should be considered in the differential diagnosis, given that TCRαβ DNT cell elevation appears to be more pronounced in patients with ALPS. FCM analysis of patients with LRBA deficiency revealed a significant reduction in switched memory B cells and expanded $CD21^{lo}$ B cells in all cases. While these B-cell disturbances are not specific, they offer valuable insights for diagnosing LRBA deficiency in patients with recurrent infections, features of immune dysregulation, and hypogammaglobulinemia (58).

Flow cytometric detection of perforin in NK cells has proven to be a rapid and sensitive test for identifying perforin deficiency (20). Typically, NK cells from patients with perforin deficiency exhibit either absent or markedly decreased perforin expression (20, 59). However, it is important to note that normal perforin expression does not definitively exclude defects in function or the presence of structurally abnormal proteins (20). In our laboratory, we have identified a patient displaying a perforin expression defect indicative of potential perforin deficiency. Remarkably, this investigated patient exhibited a strikingly normal percentage of positive NK cells (97%). However, he displayed a MFI that was notably tenfold lower compared to the control. Therefore, in the analysis of perforin expression, a comprehensive assessment should consider both the frequency and MFI for a more nuanced interpretation (60).

The DHR assay, measuring the NADPH oxidase activity, is a rapid and sensitive screening test for CGD (61). This assay can differentiate between X-CGD and AR-CGD (mainly caused by $p47^{phox}$ defect) on the basis of distinctive DHR histogram SI and

pattern (62–64). Vowells et al. demonstrated that the geometric mean SI from patients with CGD with defective gp91^{phox} and p47^{phox} were 1.3 (range, 0.9 to 2.2) and 13.2 (range, 3.5 to 52.1), respectively (61). In our cohort, three male patients displayed a complete lack of oxidative activity, indicating an XL form, while an additional four patients manifested a modest deviation in the DHR histogram with a SI ranging from 6 to 54, suggestive of a potential AR form. However, it is worth mentioning that this dichotomy is not consistently observed, as certain autosomal recessive forms, especially p22^{phox} deficiency, may present with null oxidase activity, and patients with confirmed X-CGD may exhibit a residual oxidase activity. In such instances, conducting the DHR assay for potential carriers (mothers) is crucial to identify mosaicism and predict the pattern of disease inheritance, although XL-CGD cases due to *de novo* mutation have been reported (65, 66). Additionally, further flow cytometric analysis of NADPH oxidase enzyme subunits (i.e., gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox}) in patients and carrier mothers is useful in predicting the defective gene and enabling targeted genetic sequencing, thereby allowing for a rapid and cost-effective diagnosis of CGD (66).

Based on our experience, FCM analysis has proved to be effective in diagnosing CD46 and CD55 deficiencies. Among patients with aHUS, seven exhibited either absent or significantly decreased CD46 expression suggesting both AR and AD forms of the disease. In addition, six patients presenting with PLE displayed complete loss of CD55 expression, all of whom carried biallelic mutations in the CD55 gene. In the context of surface complement protein detection, both percentages and MFI of CD46/CD55 expression were analyzed. The reference MFI values were established from a group of 10 healthy subjects. Homozygous-deficient patients exhibited MFIs comparable to the isotypic control, whereas those with heterozygous deficiency demonstrated an MFI approximately 50% of the mean MFI of the control group. Unlike some X-linked disorders such as WAS, XLA, and X-linked HIMS, CD46/CD55 heterozygous carriers did not display a bimodal expression pattern on their leukocytes. Rather, they exhibited monomodal distribution with intermediate fluorescence intensity compared with healthy controls (67–69) (Figure 5).

Over the past 7 years, our laboratory has enthusiastically embraced a diverse array of phenotyping and functional assays. While significant progress has been made, we recognize that there remain areas where our testing capabilities can be further enhanced. Therefore, we are dedicated to enhancing our testing panel by introducing new phenotypic and functional assays. Specifically, we aim to incorporate new markers such as TCRα signature (TCR Vα7.2) in V(D)J recombination defects, CD57 as a marker of senescent lymphocytes in APDS, gp91^{phox}, p22^{phox}, p47^{phox}, and p67^{phox} in CGD, as well as signaling lymphocyte activation molecule (SLAM)–associated protein (SAP) and X-linked inhibitor of apoptosis protein (XIAP) in X-linked lymphoproliferative diseases 1 and 2 (12, 66, 70, 71). Additionally, we aspire to integrate lymphocyte proliferation assays into routine practice and develop new functional tests, such as the assessing of histone H2AX phosphorylation (γH2AX) in CD3 T cells following irradiation, commonly used in diagnosing Ataxia Telangiectasia and radiosensitive SCIDs (72, 73).

5 Conclusion

Flow cytometry has emerged as a highly valuable and cost-effective tool for diagnosing and studying IEIs, particularly in low-income countries where access to genetic testing is limited. A stratified flow cytometric analysis of specific proteins or particular subpopulations plays a crucial role in establishing definitive diagnoses for various disorders. While molecular testing remains necessary for a definitive diagnosis in some IEI disorders, conducting phenotypic or functional analyses of lymphocyte subsets provides clues to the underlying genetic defects or aids in narrowing down the list of putative genes to be analyzed.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRJNA1089953 (SRA).

Ethics statement

The studies involving humans were approved by Ethics committee of Rouiba Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

AT: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Writing – original draft. RBe: Data curation, Writing – review & editing. AY: Data curation, Investigation, Writing – review & editing. SH: Data curation, Writing – review & editing. FF: Data curation, Writing – review & editing. MK: Data curation, Writing – review & editing. HBe: Data curation, Writing – review & editing. ST: Data curation, Writing – review & editing. SA: Data curation, Writing – review & editing. JS: Data curation, Writing – review & editing. JN: Data curation, Writing – review & editing. FZ: Data curation, Writing – review & editing. SM: Data curation, Writing – review & editing. RA: Writing – review & editing. AS: Writing – review & editing. YF: Writing – review & editing. AK: Writing – review & editing. HMe: Writing – review & editing. TB: Data curation, Writing – review & editing. ZBe: Data curation, Writing – review & editing. AD: Writing – review & editing. KO: Writing – review & editing. FA: Data curation, Investigation, Writing – review & editing. MF: Writing – review & editing. CBe: Data curation, Writing – review & editing. RK: Data curation, Writing – review & editing. AO: Data curation, Writing – review & editing. AS: Writing – review & editing. IB: Writing – review & editing. CBo: Writing – review & editing. NBo: Writing – review & editing. IM: Writing – review & editing. NK: Writing – review & editing. HBo: Software, Writing – review & editing. TK: Writing – review & editing. FM: Writing – review & editing. MBou: Writing – review & editing. AZ: Writing – review & editing. OG: Writing – review & editing. ML: Writing – review & editing. AM:

Writing – review & editing. NBe: Writing – review & editing. MBen: Writing – review & editing. ZZ: Writing – review & editing. BB: Data curation, Investigation, Writing – review & editing. MuB: Writing – review & editing. BA: Writing – review & editing. ZBo: Writing – review & editing. OI: Writing – review & editing. HMa: Data curation, Investigation, Writing – review & editing. LK: Data curation, Investigation, Writing – review & editing. LS: Writing – review & editing. RBouk: Supervision, Writing – review & editing. CL: Data curation, Investigation, Writing – review & editing. SR: Data curation, Investigation, Writing – review & editing. LN: Data curation, Investigation, Writing – review & editing. KD: Data curation, Investigation, Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Acknowledgments

We would like to thank Dr Dusan Bogunovic for his contribution to the identification of genetic defects in our patients and for the critical review of the manuscript. We express our deepest gratitude for the patients and their families to whom our work is dedicated.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1402038/full#supplementary-material

SUPPLEMENTARY FIGURE 1
IEI distribution in our series. ALPS, autoimmune lymphoproliferative syndrome; APDS, activated PI3K delta syndrome; ARPC1B, actin related protein 2/3 complex subunit 1B; CGD, chronic granulomatous disease; CID, combined immunodeficiency; CHAPLE, complement hyperactivation angiopathic thrombosis and protein-losing enteropathy; CMC, chronic mucocutaneous candidiasis; CVID, common variable immunodeficiency; fHLH, familial hemophagocytic lymphohistiocytosis; HIES, hyper-IgE syndrome; IPEX, immune dysregulation-polyendocrinopathy-enteropathy-x-linked; LAD, leukocyte adhesion deficiency; LRBA, LPS-responsive beige-like anchor protein; MHC, major histocompatibility complex; MSMD, mendelian susceptibility to mycobacterial disease, OS, Omenn syndrome; PAD, predominantly antibody deficiencies; RIPK1, receptor-interacting serine/threonine-protein kinase 1; SCID, severe combined immunodeficiency; SIAD, selective IgA deficiency; TPP2, tripeptidyl peptidase 2; STAT, signal transducer and activator of transcription.

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 24 July 2024
DOI 10.3389/fimmu.2024.1430938

OPEN ACCESS

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RECEIVED 10 May 2024
ACCEPTED 13 June 2024
PUBLISHED 24 July 2024

CITATION
Fink F-M, Höpfl R, Witsch-Baumgartner M, Kropshofer G, Martin S, Fink V, Heeg M, Peters C, Zschocke J and Haas OA (2024) Retrospective identification of the first cord blood–transplanted severe aplastic anemia in a *STAT1*-associated chronic mucocutaneous candidiasis family: case report, review of literature and pathophysiologic background. *Front. Immunol.* 15:1430938. doi: 10.3389/fimmu.2024.1430938

Retrospective identification of the first cord blood–transplanted severe aplastic anemia in a *STAT1*-associated chronic mucocutaneous candidiasis family: case report, review of literature and pathophysiologic background

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Severe aplastic anemia (SAA) is a life-threatening bone marrow failure syndrome whose development can be triggered by environmental, autoimmune, and/or genetic factors. The latter comprises germ line pathogenic variants in genes that bring about habitually predisposing syndromes as well as immune deficiencies that do so only occasionally. One of these disorders is the autosomal dominant form of chronic mucocutaneous candidiasis (CMC), which is defined by germ line *STAT1* gain-of-function (GOF) pathogenic variants. The resultant overexpression and constitutive activation of STAT1 dysregulate the Janus kinase/signal transducer and activator of transcription 1 (STAT) signaling pathway, which normally organizes the development and proper interaction of different components of the immunologic and hematopoietic system. Although SAA is an extremely rare complication in this disorder, it gained a more widespread interest when it became clear that the underlying causative pathomechanism may, in a similar fashion, also be instrumental in at least some of the idiopathic SAA cases. Based on these premises, we present herein what is the historically most likely first cord blood–transplanted SAA case in a CMC family with a documented *STAT1* GOF pathogenic variant. In addition, we recapitulate the characteristics of the six CMC SAA cases that have been reported so far and discuss the significance of *STAT1* GOF pathogenic variants and other STAT1

signaling derangements in the context of these specific types of bone marrow failure syndromes. Because a constitutively activated STAT1 signaling, be it driven by *STAT1* GOF germ line pathogenic variants or any other pathogenic variant-independent events, is apparently important for initiating and maintaining the SAA disease process, we propose to acknowledge that SAA is one of the definite disease manifestations in *STAT1*-mutated CMC cases. For the same reason, we deem it necessary to also incorporate molecular and functional analyses of *STAT1* into the diagnostic work-up of SAA cases.

KEYWORDS

chronic mucocutaneous candidiasis (CMC), severe aplastic anemia (SAA), STAT1, gain-of-function pathogenic variant, transplantation

Introduction

Severe aplastic anemia (SAA) is a life-threatening bone marrow failure (BMF) syndrome that can be caused by a radiation-evoked damage of the hematopoietic system, an autoimmune- or infection-associated destruction of bone marrow cells either by hepatitis B, parvo-, cytomegalo-, or Epstein–Barr viruses or by other yet unknown causes (1). There are three genetic disorders that typically predispose affected children to the development of SAA, namely, the Fanconi anemia DNA repair and dyskeratosis congenita telomere maintenance systems and the Shwachman–Diamond syndrome, a disorder of defective ribosome biogenesis (2–5). Moreover, SAA may occasionally also develop in the context of four specific immune disorders. These include the adenosine deaminase 2 deficiency (due to homozygous or compound heterozygous variants in the *ADA2/CERC* gene) (6–8), the loss of function (LOF) of the cytotoxic T-lymphocyte–associated protein 4 (caused by autosomal recessive variants in the *CTLA4* gene) (4, 9), the gain of function (GOF) of the Toll-like receptor 8 (10–12), and the signal transducer and activator of transcription 1 (STAT1), which all result from dominant heterozygous gene alterations (13–16).

Recurrent and persistent infections with various candida species are the hallmark of several genetically distinct immunodeficiency disorders (17–24). The autosomal dominant form of chronic mucocutaneous candidiasis (CMC) results from *STAT1* GOF pathogenic variants (OMIM #614162 and orphan designation ORPHA1334) (18, 19, 22, 24–26). They designate neither a genetically nor clinically distinct entity but rather a form of combined immunodeficiency with recurrent or persistent infections of the nails, skin, and mucous membranes that are caused by various candida species and commonly accompanied by diverse other bacterial and viral infections as well as a smorgasbord of autoimmune and inflammatory disorders, cytopenias, aneurysms, and squamous cell carcinomas (19, 22, 26, 27). The most frequent autoimmune manifestations comprise thyroiditis and destructive blood cytopenias, such as autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), and neutropenia. The few cases of SAA in carriers with *STAT1* GOF variants gained a more widespread interest when it became clear that also pathogenic variant-independent modes of STAT1 activation can trigger the development of idiopathic SAA forms, an insight that is, nowadays, already exploited therapeutically with drugs that inhibit the Janus kinase (JAK) (13–16, 28–31).

We report herein a CMC family with three *STAT1* pathogenic variant carriers, two brothers and their father, who had been transplanted as a child because of a SAA.

Family history

A 4-year-old boy (patient 1) presented with CMC-associated recurring oral candidiasis and candida-associated paronychia since infancy, body-wide scaly seborrheic eczemas that extended to the eyelids, and a dystrophic growth of both thumbnails due to a chronic inflammation of the nail folds (Figure 1). His 14-month-old brother (patient 2) developed a severe oral thrush 3 months later (Figure 1D). In 1991, 28 years earlier, their father (patient 3) developed a SAA when he was 4 years old. At that time, his white blood cell count was 3.6×10^9/L (age-adjusted normal range, 4.1 to 14.6×10^9/L) with an absolute neutrophil count of 324×10^6/L (normal, 200×10^6/L) and a red blood cell count of 1.08×10^{12}/L (normal, 3.98 to 5.33×10^9/L). His hemoglobin was 3.6 g/dL (normal, 10.7 g/dL to 14.2 g/dL) and his platelets were 10×10^9/L (normal, 168 to 453×10^9/L) (32). Eleven months earlier, he had a hepatitis A infection; 6 months earlier, a bronchitis; and 2 months, earlier a whooping cough. When he was 3 years old, he had developed a mild psoriasis-like seborrheic eczema but none of the more specific CMC signs. The patient became transfusion-dependent 2 months after first presentation and was, therefore, treated unsuccessfully with high-dose corticosteroids and anti-lymphocyte globulin. Six months later, he was transplanted with cord blood cells from his newly born brother. The conditioning consisted of cyclophosphamide at 200 mg/kg and 5 Gy of total nodal irradiation and the graft-versus-host prophylaxis of ciclosporin A and methotrexate. Stem cell engraftment was delayed, but, following G-

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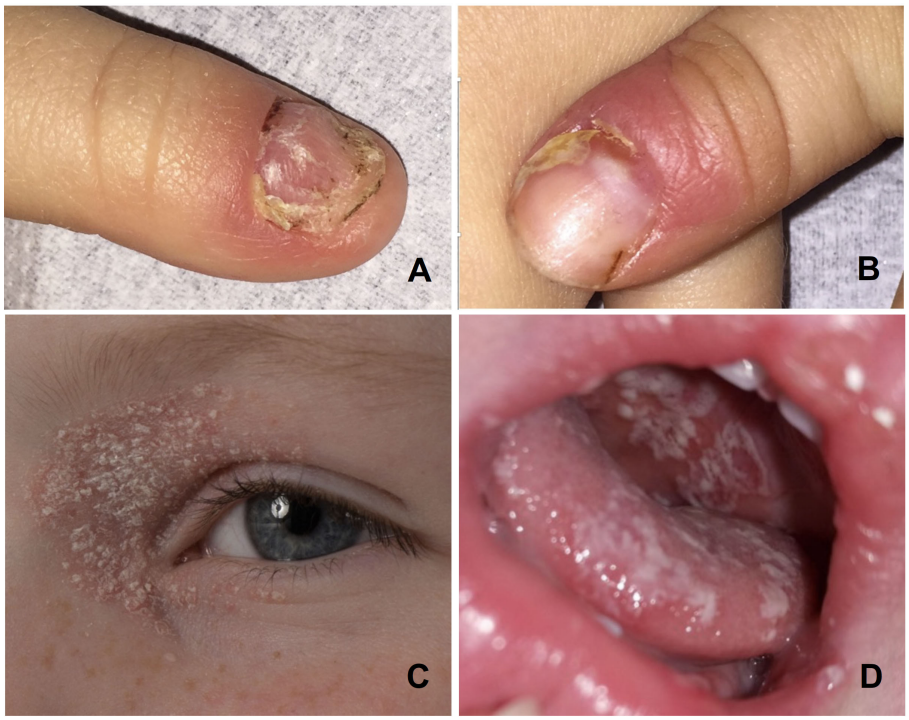

FIGURE 1
Photographs of the CMC manifestations in the two boys: Candida paronychias and onychodystrophic thumbnails **(A, B)** and periocular seborrheic eczema **(C)** in the index patient 1, and oral candidiasis in his brother **(D)**.

CSF treatment, his white blood cell count rose over $1.0 \times 10^3/\mu L$ on day +39. He received his last platelet transfusion on day +40 and the last unit of packed red blood cells on day +47. Since then, the now 37-year-old father has normal blood cell counts, had never experienced any CMC symptoms, and remained healthy. Also, the clinical course of the two affected boys is so far benign. They require only occasionally symptomatic antifungal treatment. Fluorescence activated cell sorting (FACS) analyses of peripheral blood mononuclear cells of the two brothers and their transplanted father revealed a normal distribution of lymphocyte subpopulations. Their levels of immunoglobulins and immunoglobulin G (IgG) subclasses were normal and none of them had developed autoantibodies or endocrine deficiencies.

Genetic analyses

To identify the genetic cause of this familial disorder, we used the Illumina's TruSight™ One Panel to sequence DNA that was extracted from the older boy's peripheral blood cells and focused our screening efforts on the three most likely responsible genes, namely, *STAT1*, *IL17RA*, and *AIRE*. This approach uncovered a hitherto undescribed heterozygous *STAT1* missense variant c.1013G>T (p.Gly338Val) that affects the DNA-binding domain (Figure 2) (19). Based on the criteria put forward by the American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants, we initially classified it as variant of unknown significance (33). Twenty-eight years after the father had been transplanted, we succeeded to identify this variant in his skin fibroblasts with an allele frequency of 50%. Neither the children's parental grandparents nor their mother carried the pathogenic variant. The fact that we found the same variant in both diseased brothers and their father enabled us to reclassify it as likely pathogenic.

Functional evaluation of the *STAT1* pathogenic variant

To further verify the functional relevance of this novel *STAT1* missense variant, we stimulated peripheral blood mononuclear cells (PB-MNCs) of the two brothers with interferon (IFN)–α and IFN-γ, which led to a significant increase in the phosphorylation of STAT1 (Figure 3). Conversely, incubating PB-MNCs with the "nuclear factor 'kappa-light-chain enhancer' of activated B cells" (NF-κB) activators phorbol myristate acetate and ionomycin disclosed an impaired expression of interleukin-17 (IL-17) as well as IFN-γ in the patient's CD45RO+CD4+ T cells (Figure 3). Together, these results clearly proved the GOF nature of this c.1013G>T *STAT1* missense variant.

Discussion

The most remarkable finding in the CMC family reported herein is that the father was a *STAT1* GOF variant carrier (case 7,

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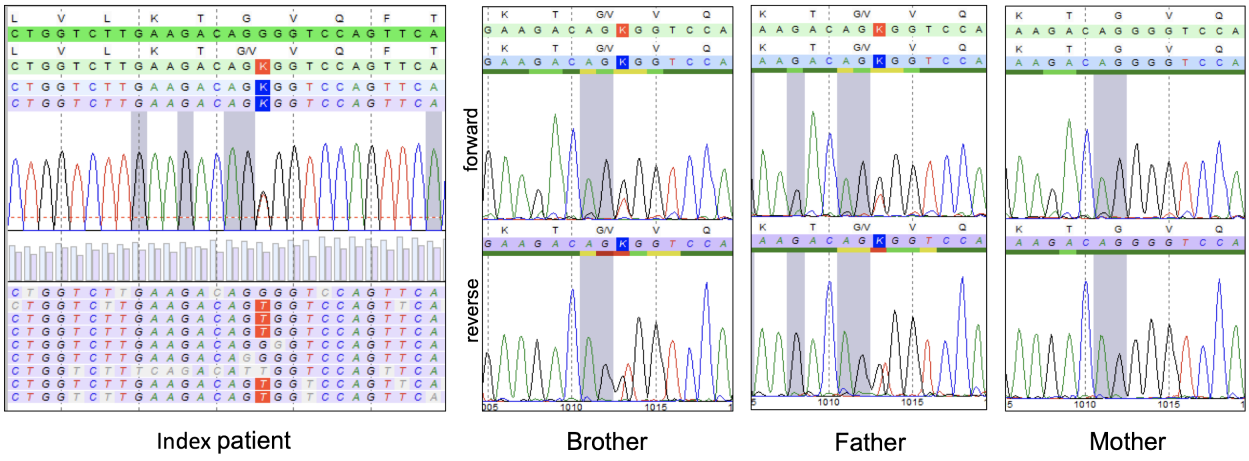

FIGURE 2
Result of the pathogenic variant screening in our CMC family. In the index patient, targeted pathogenic variant screening revealed a heterozygous c.1013G>T (p.Gly338Val) missense pathogenic variant in the *STAT1* gene, which was subsequently also identified in the peripheral blood of his brother and in the fibroblasts of his father with Sanger sequencing the respective PCR-amplified Exon 11, but not in the healthy mother.

Table 1). As a child, he was transplanted with his brother's cord blood cells because of a presumably infection- or autoimmune-induced SAA. If it had not been for his diseased sons, then we would have never contemplated that he might be a *STAT1* GOF variant carrier. Yet, 28 years later, we were able to secure the GOF variant in his skin fibroblasts and, by that, identify the historically first SAA case with a *STAT1* GOF variant who had received a life-saving cord blood transplant.

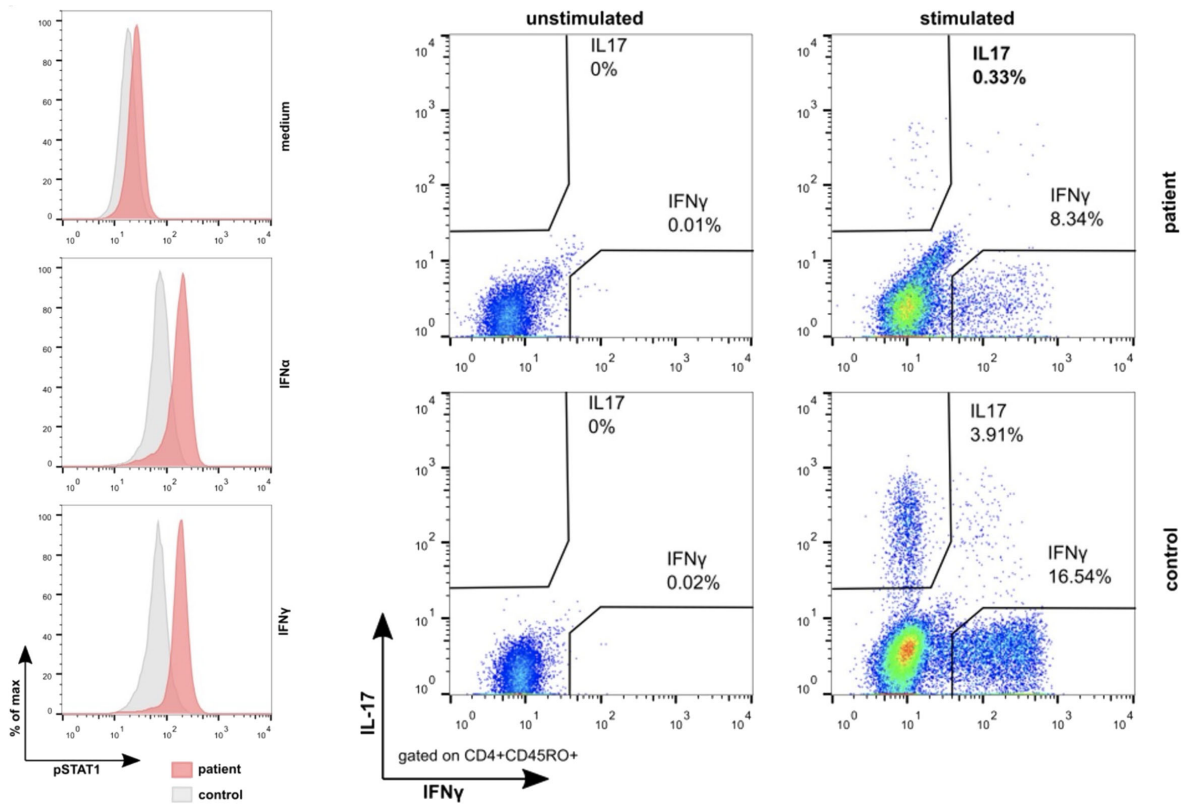

FIGURE 3
Results of the analyses of the functional consequences of the *STAT1* c.1013G>T (p.Gly338Val) missense pathogenic variant. Compared to healthy controls, stimulation with interferon-α as well as interferon-γ leads to the hyperphosphorylation of STAT1 in the monocytes (left) of the index patient and his brother (not shown). Conversely, stimulation with the NF-κB activator phorbol myristate acetate and ionomycin revealed the impaired expression of interleukin-17 as well as interferon-γ in their CD45RO+CD4+ T cells. Together, these findings provide persuasive evidence for the GOF effect of this pathogenic variant.

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TABLE 1 Overview of seven cases with CMC, GOF-STAT1 mutations and SAA.

Case	Sex	Age (years)	Clinical picture	*STAT1 Pathogenic variant*	Location (domain)	Treatment	Reference
1	Male	56	CMC, pernicious anemia, thymoma, SAA	not available	-	Plasmapheresis	(34)
2	Female	7	CMC, SAA	not available	–	Bone marrow transplantation (1982)	(35)
3	Female	10	CMC, AIHA, ITP, suspected MAS, SAA	c.1633G>A (p.Glu545Lys)	Linker	ATG + CyA, JAK1/2-inhibitor (ruxolitinib)	(16)
4	Male	18	CMC, SAA	c.800C>/T (Ala267Val)	Coiled coil	JAK1-inhibitor (icatinib)	(15)
5	Female	8	CMC, SLE, AIHA, hypothyroidism, PRCA	c.854A>G (p.Q285R)	Coiled coil	JAK1/2-inhibitor (ruxolitinib), stem cell transplantation	(14)
6	Female	32	no CMC, oral ulcers, Typ-1 diabetes, SAA	c.520T>C (p.Cys174Ala)	Coiled coil	Not reported	(13)
7	Male	4	no CMC, SAA	c.1013G>T (p.Gly338Val)	DNA binding	Cord blood transplantation (1991)	Present report (father)

AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia; MAS, macrophage activating syndrome; SLE, systemic lupus erythematosus; ATG, anti-thymocyte globulin; CyA, ciclosporin A.

In individuals with CMC, genuine BMF syndromes, such as SAA and pure red cell aplasia (PRCA), seem either extremely rare or underreported (19, 22, 26, 36). There are only two such cases that were published earlier. The first one was reported in 1975 by Twomey et al. (case 1, Table 1) (34). An already 56-year-old man developed SAA and a small thymoma 9 years after mucocutaneous candidiasis was first noted. The second case concerned a 7-year-old girl with CMC and SAA, who was successfully treated with a bone marrow transplant (case 2, Table 1) (35).

In addition to these two cases and the one presented herein, only three other *STAT1*-associated CMC cases with SAA and one with a PRCA were reported (Table 1) (13–16). The first of these concerns a 10-year-old girl who originally suffered from a life-threatening Evans syndrome with episodes of AIHA and ITP (case 3, Table 1) (16). Later on, she developed a SAA together with a suspected macrophage activation syndrome. She was treated with steroids, intravenous immunoglobulins, the anti-CD20 antibody rituximab, the anti-complement C5 antibody eculizumab, and, thereafter, with anti-thymocyte globulin and ciclosporin A. Only the latter restored her bone marrow function to some extent but did not produce a complete remission. Following the detection of a *STAT1* GOF pathogenic variant and the subsequent meticulous *in vitro* assessment of its functional consequences, she received the JAK1/2 inhibitor ruxolitinib. Continuous treatment with this inhibitor alone resolved all her autoimmune-mediated problems, and she was reported to be still in complete remission 18 months later.

The second case concerns an 18-year-old man with oral ulcers and CMC who developed SAA with an only moderately decreased number of platelets, but weekly transfusion-dependent anemia and several episodes of febrile neutropenia (case 4, Table 1) (15). He was treated with the JAK1 inhibitor itacitinib for 20 months without any adverse effects that resulted in a rapid and remarkable recovery of hematopoiesis, before he self-discontinued this treatment. Three years after initial presentation and approximately 12 months off itacitinib therapy, he was still in continuous hematologic remission. Based on the discovery and insight that a significant proportion of other patients with "idiopathic" forms of SAA share similar STAT1-mediated pathophysiologic changes, the authors were the first to suggest that such "idiopathic" cases might also benefit from treatment with JAK inhibitors in a similar manner.

The third case concerns a 32-year-old woman with oral ulcers, oral candidiasis, recurrent pneumonia, type 1 diabetes mellitus, and SAA with a relatively preserved thrombocyte level (case 5, Table 1) (13). The authors did not provide any further clinical information, but they also found that STAT1 can be overexpressed in patients with idiopathic SAA.

Because PRCA and SAA are BMF syndromes with a closely related etiology, we also include the girl with CMC and a PRCA (case 6, Table 1) (14). With 1 year, she already suffered from a systemic lupus erythematosus before she developed CMC-typical infections and eventually PRCA with 8 years. Because conventional immunosuppressive therapies were not effective, she received the JAK inhibitor ruxolitinib and was transplanted after she had transiently improved but succumbed to transplant-related complications.

STAT1 encodes one of the seven STAT family transcription factors. They are downstream components of the JAK signaling pathway, which regulates the expression of more than 50 cytokines, growth factors and IFNs (37). After binding to their cognate receptors, the respective ligands dimerize and activate JAKs, which phosphorylate the STAT proteins and, by that, turn on their individual transcriptional programs (15, 37, 38). An activated STAT1 not only impacts the expression of other transcription factors but also self-regulates its own expression (39).

The constantly enhanced STAT1 signaling in *STAT1* GOF variant carriers creates two closely interlinked feedback loops that enhance the IFN-controlled and inhibit the IL-17–controlled immune defense (25, 40–47). Within this context, STAT1 acts both as a signal transducer and transcription activator for the IFN type I (IFN-α and IFN- β), type II (IFN-γ), and type III (IFN-λ) as well as for IL-27 (25, 40, 41, 48). The increased levels of these factors eventually cause all the varied inflammatory, autoimmune, and tissue destructive ailments that CMC patients

have to endure (19, 22, 40, 41). The fact that STAT1 signaling occurs through both the type I and the type II IFN receptors prompted Largent et al. to examine the differences between these two transmission routes (43). They found that the deletion of the type I IFN receptors in mice with a *Stat1* GOF pathogenic variant only partly protected them from STAT1-driven systemic inflammations, whereas deletion of the type II IFN receptors normalized total STAT1 expression, which safeguarded them from autoimmune manifestations (43). These findings suggest that IFN-γ is the relevant driver that upregulates STAT1 in a *STAT1* GOF environment (41, 43). The long-term exposure of hematopoietic stem cells to high IFN-γ levels inhibits their self-renewal capacity and pushes them toward terminal differentiation (49–52). It is, therefore, conceivable that an exhaustion of the stem cell compartment is one of the reasons why, in some *STAT1* GOF variant carriers, the bone marrow will fail. We presume that, in our SAA case, this process was most likely instigated and reinforced by the three infections that he had gone through beforehand.

Whether and to which extent STAT1 activity is primarily governed alone by an enhanced production, by the subsequent phosphorylation, and/or by an impaired dephosphorylation of the respective proteins is still a controversial and unresolved issue. While an *in vitro* IFN-α and IFN-γ stimulation–associated phosphorylation is a diagnostic hallmark of every *STAT1* GOF variant (46), Scott et al. and others suggested that STAT1 increased phosphorylation might be a secondary event and only take place when the STAT1 level is already elevated. They, therefore, consider the overall amount of unphosphorylated STAT1 as probably being more relevant for the primary pathogenic effects than phosphorylation itself (39, 53, 54).

Recent *in vitro* experiments provided some evidence that specific *STAT1* GOF variants may predetermine specific disease patterns and clinical phenotypes (25, 39, 55, 56). This notion derives from the variant-specific IFN-α and IFN-γ gene expression patterns that were caused by two different *STAT1* GOF variants in a STAT1-deficient U3A fibrosarcoma cell line transfection model (56). These observations were later extended by Scott et al., who generated five heterozygous CRISPR/Cas9 base–edited *STAT1* GOF variants in diploid HAP1 cells and found that baseline as well as IFN-α or IFN-γ induced expression levels of five investigated IFN-stimulated genes varied starkly depending on the specific pathogenic variant (39). Taken together, these widely divergent variant-specific transcriptional responses suffice at least to comprehend why the resultant disease spectra are so diverse and why it is virtually impossible to predict the overall clinical course in *STAT1* GOF–mutated individuals.

The second feedback loop revolves around the highly versatile context- and tissue-dependent function of the proinflammatory cytokine IL-17 (42, 47, 57). IL-17 is primarily protective against fungal as well as, to a lesser extent, also other types of mucocutaneous infections, which is the reason why individuals with an insufficient IL-17 defense develop such otherwise uncommon diseases (21, 42, 46, 57, 58). However, even in cases with an intact IL-17 response, the local effects of an IFN-γ/STAT1–driven interferonopathy alone can be enough to promote mucocutaneous fungal infections by impairing the integrity of the epithelial barrier (42, 57, 59). Because, in *STAT1* GOF–mutated individuals, both these immune signaling alterations usually concur, their effects may jointly contribute to their heightened susceptibility and the severity of fungal infections.

A hyperactive STAT1 downregulates the IL-17 signaling pathway by interfering with the STAT3-mediated differentiation program of Th17 cells, as already evidenced by the similar immunological and clinical effects of *STAT3* LOF and *STAT1* GOF variants (41, 46, 60–62). Activation of STAT1 and STAT3 by IL-6, IL-21, IL-27, and other factors affect the differentiation program of Th17 cells in opposing ways that range from strong induction (IL-6) to strong inhibition (IL-27) (21, 47, 61, 63, 64). This tightly STAT1- and STAT3-regulated cellular differentiation and interaction network relies on a well-balanced and appropriately adjusted intracellular ratio of the STAT1 and STAT3 transcription factors. Because STAT1 and STAT3 form homo- as well as heterodimers, an overabundance of STAT1 will upregulate the STAT1 transcription program either in a direct or indirect way, for instance, by competing with STAT3 for common receptor docking sites on their respective target promotors (41, 62, 65).

Noteworthy, IL-27 priming impairs also Th17 differentiation by upregulating the programmed cell death protein ligand 1 (PD-L1) in CD4-positive T cells (66, 67). Because this occurs in a STAT1-dependent manner, PD-L1 expression is markedly increased in STAT3 LOF and *STAT1* GOF naïve $CD4^+$ T cells. Although such Th17 differentiation defects might theoretically be overcome by blocking PD-L1 with checkpoint inhibitors, this is currently not a viable option in auto-immune diseases because of adverse effects of the respective drugs (66, 68, 69).

Patients with CMC with a *STAT1* GOF pathogenic variant are at risk to develop a broad spectrum of infectious and non-infectious complications. The first sign in infants is usually a recurrent or persisting oral candidiasis (thrush) alone or in combination with chronic nail-fold inflammation. As long as the course of CMC is mild and remains controllable, symptomatic treatment with antifungal, antibacterial, antiviral, and perhaps immune-dampening remedies is generally sufficient (17, 70). The only potential curative option for cases with more severe, exacerbating, and life-threatening disease forms was, for a long time, a hematopoietic stem cell transplantation. With a high rate of secondary graft failures and survival rates of only up to 40%, the overall outcome of this procedure is still poor (26, 36, 71). The main arguments that are commonly put forward to explain these disappointing outcome data are the advanced disease stage and consequently also the poorer health state of patients who are selected for transplantation (36, 71). Thus, some of the factors, which assured that the transplantation went smoothly and without complications in our patient, were his young age, his overall good clinical condition, and the lack of any noteworthy additional ailments before he received his cord blood graft.

The identification of *STAT1* pathogenic variants together with their subsequent meticulous functional assessment paved the way for the development for well-defined targeted treatment approaches (17). The currently best explored ones are JAK inhibitors, such as the JAK1/2 inhibitor ruxolitinib. It downregulates JAK signaling and thereby also reduces STAT1 activity (14–16, 31, 72–78). Hence, JAK inhibitors help to manage the disease by suppressing autoimmune processes and by improving host defense mechanisms. Although sufficient data on the risk-benefit ratio, especially on the long-term application are still

pending, JAK inhibitors are already increasingly used in experienced treatment centers in an off-label setting for bridging or as an alternative for stem cell transplantation (72, 73, 77). Another potentially interesting drug that could become helpful in the pretransplant preparation of patients with STAT1 GOF pathogenic variants is the IFN-γ blocker emapalumab (79).

Taken together, we presented herein what is the historically first cord blood–transplanted SAA case in a CMC family with a *STAT1* GOF pathogenic variant. We reviewed the characteristics of all available six cases with CMC and a BMF and briefly discussed the role of *STAT1* GOF pathogenic variants and other STAT1 signaling derangements that are encountered in pathogenic variant-driven and sporadic SAA cases, respectively. Based on this information, we deem it necessary to acknowledge that SAA is one of the definitive hematologic manifestations of *STAT1* GOF–associated CMC and to perform molecular and functional analyses of *STAT1* in all unclear cases of BMFs in the future.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving human samples in accordance with the local legislation and institutional requirements because all the molecular-genetic analyses, functional studies and therapeutic interventions were part of the advanced standard care of the involved patients. Written informed consent for participation in this study was provided by the participants' legal guardians/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. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

F-MF: Writing – original draft, Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. RH: Writing – review & editing, Supervision, Investigation, Data curation, Funding acquisition. MW-B: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. GK: Writing – review & editing, Data curation. SM: Writing – review & editing, Data curation. VF: Writing – review & editing, Data curation. MH: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. CP: Writing – review & editing, Data curation. JZ: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition. OH: Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation, Conceptualization.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. We are grateful for the financial support provided by the St. Anna Children's Cancer Research Institute (CCRI) Vienna, the Department of Dermatology and Venerology and the Institute of Human Genetics, Medical University Innsbruck, to cover the publication costs.

Acknowledgments

We acknowledge the excellent support of the CCI Advanced Diagnostic Unit (Ilka Fuchs) and the CCI Clinical Research Unit/Biobank (Alexandra Nieters).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 06 August 2024
DOI 10.3389/fimmu.2024.1377955

OPEN ACCESS

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RECEIVED 28 January 2024
ACCEPTED 21 June 2024
PUBLISHED 06 August 2024

CITATION
Graafen L, Heinze A, Albinger N, Salzmann-Manrique E, Ganß F, Hünecke S, Cappel C, Wölke S, Donath H, Trischler J, Theilen T-M, Heller C, Königs C, Ehl S, Bader P, Klingebiel T, Klusmann J-H, Zielen S, Schubert R and Ullrich E (2024) Immune profiling and functional analysis of NK and T cells in ataxia telangiectasia. *Front. Immunol.* 15:1377955. doi: 10.3389/fimmu.2024.1377955

Immune profiling and functional analysis of NK and T cells in ataxia telangiectasia

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Ataxia telangiectasia (AT) is a rare autosomal-recessive disorder characterized by profound neurodegeneration, combined immunodeficiency, and an increased risk for malignant diseases. Treatment options for AT are limited, and the long-term survival prognosis for patients remains grim, primarily due to the emergence of chronic respiratory pathologies, malignancies, and neurological complications. Understanding the dysregulation of the immune system in AT is fundamental for the development of novel treatment strategies. In this context, we performed a retrospective longitudinal immunemonitoring of lymphocyte subset distribution in a cohort of AT patients (n = 65). Furthermore, we performed FACS analyses of peripheral blood mononuclear cells from a subgroup of 12 AT patients to examine NK and T cells for the expression of activating and functional markers. We observed reduced levels of peripheral blood $CD3^+CD8^+$ cytotoxic T cells, $CD3^+CD4^+$ T helper cells, and $CD19^+$ B cells, whereas the amount of $CD3^{-}CD56^+$ NK cells and $CD3^+CD56^+$ NKT-like cells was similar compared with age-matched controls. Notably, there was no association between the age-dependent kinetic of T-, B-, or NK-cell counts and the occurrence of malignancy in AT patients. Additionally, our results indicate an altered NK- and T-cell response to cytokine stimulation in AT with increased levels of TRAIL, FasL, and CD16 expression in NK cells, as well as an elevated activation level of T cells in AT with notably higher expression levels of IFN-γ, CD107a, TRAIL, and FasL. Together, these findings imply function alterations in AT lymphocytes, specifically in T and NK cells, shedding light on potential pathways for innovative therapies.

KEYWORDS

ataxia telangiectasia, NK cells, T cells, immune characterization, phenotyping

Highlights

- In a large cohort of AT patients (n = 65), we observed reduced levels of B cells, $CD4^+$ cells, and $CD8^+$ T cells and no difference in the amount of NK and NKT cells in peripheral blood compared with age-matched reference values.
- Longitudinal evaluation of immune cell counts in the same AT cohort did not predict the subsequent development of malignancies.
- Extended immune phenotyping indicates altered expression of death receptor ligands and CD16 in stimulated NK cells together with a higher activation level of T cells in AT, demonstrated in the increased expression of IFN-γ, CD107a, TRAIL, and FasL.

Introduction

Ataxia telangiectasia (AT) is a rare autosomal recessive disorder, presenting with severe neurodegeneration, immunodeficiency, a considerably increased risk for malignant diseases, and a devastating prognosis (1). In the disease pathogenesis, a genetic defect in *ataxia-telangiectasia-modified*-gene (*ATM*-gene) on chromosome 11q22–23 leads to dysfunction of the ataxia-telangiectasia-modified protein (ATM-protein) (1), a kinase mainly involved in the repair of DNA-double-strand breaks (2, 3). Defects in the ATM-protein have impact on important immunological functions such as cell cycle control (4), meiotic recombination, and recombination in immunoglobulin genes, which are important for the rearrangement of the B- and T-cell receptor (BCR; TCR) genes and class switch of mature B cells (1, 5, 6).

There are two major distinct AT phenotypes differing in disease progress. While *classical AT* patients show a complete loss of ATM functionality and usually present with high morbidity, in *variant AT* patients, a certain functionality of the ATM protein can be observed, leading to a milder clinical course of disease and longer survival (7–9). In addition, patients with *classical AT* with IgA deficiency have significantly lower lymphocyte counts and subsets, which are accompanied by reduced survival, compared with AT patients without IgA deficiency (10, 11).

The two major causes of death in AT are malignant diseases and chronic pulmonary pathologies (1, 12, 13), which are both likely linked to the immunological dysfunctions (14, 15). In this regard, it is a key factor to understand the underlying mechanisms of immunodeficiency in AT. Up to now, our understanding indicates impairment in both the cellular and humoral immune system in affected patients. They often present with severe lymphopenia and reduced levels of IgG2, IgA, and IgE antibodies (16–19). Within the lymphocyte population, decreased numbers of T and B cells can be observed whereas an elevated number of NK cells have been suggested (16). Within the T-cell population, there is a lack of naïve ($CD45RA^+$) $CD4^+$ and $CD8^+$ T cells, whereas numbers of T memory cells ($CD45RO^+$) remain in the normal range (16, 20).

In the context of immunodeficiency in AT, recurrent infections mainly of the respiratory tract play a major role. In some cases, also granulomas can emerge, which are usually restricted to the skin (21). As a pathophysiological mechanism, a dysregulation of the immune system, involving among others macrophages, T cells, and NK cells, has been supposed and treatment with TNF inhibitors was found to be successful in some patients (21).

Since NK cells play a major role in the first line of defense against pathogens and are crucial for the recognition and elimination of malignant cells, we investigated their functionality in ATM deficiency. In parallel, T cells were studied, which are known for their elementary role in the adaptive immune response and were already reported with altered functions in AT (16). The aim was to perform a detailed cellular immune profiling of NK and T cells in AT to better understand the existing immunodeficiency.

Therefore, retrospective lymphocyte phenotyping data were collected from a total of 65 AT patients from the Department of Pediatrics at the University Hospital Frankfurt. The longitudinal course of T, B, NK, and NKT-like cells was compared with corresponding age-dependent reference values (22, 23), and the association between the immunological profile of AT patients and the occurrence of malignant diseases was studied.

For further functional FACS analysis, primary blood samples of 12 AT patients of the same cohort as well as primary blood samples of 17 healthy controls were collected and analyzed. We specifically investigated two different cytotoxicity pathways, the granzyme-perforin-dependent and the death receptor-dependent one, as well as CD107a and IFN- γ expression as markers of NK- and T-cell degranulation, cytotoxicity, and cytokine production (24). Important clinical data on this subgroup were collected retrospectively by reviewing patient records.

Overall, the identification of changes in NK- and T-cell phenotype and functionality in AT is fundamental, not only to understand the disease progress but also to possibly support developing novel treatment strategies such as supportive cell therapy concepts in the future.

Materials and methods

The study design consists of a retrospective, longitudinal analysis of immune phenotype in AT patients as well as a cross-sectional analysis of more extensive NK- and T-cell phenotyping using functional markers.

Study cohort

The study collected retrospective data from 65 AT patients of the Department of Pediatrics of the University Hospital Frankfurt. Diagnosis of AT was based on clinical and/or genetic findings. All

patients with available flow cytometry data were included, except for patients with *variant AT* phenotype or participants of pharmacological clinical studies. Collected data comprise age; gender; the absolute numbers of T subsets, NK, and B cells; the development of malignant disease and its date; and the time of decease if applicable. Measurements of immune cell subsets were performed within the framework of patient monitoring and corresponds to its clinical requirements. Observation started as early as data of the individual patients was available and was limited to the end of the year 2022.

Additionally, blood samples from 12 of the 65 AT patients and 17 immunological healthy controls were collected in the cross-sectional study. Blood samples of AT patients were collected at one timepoint during routine check-up examinations between October 2018 and January 2020, whereas immunological healthy individuals presented for minor elective surgery or for consultation in the pediatric coagulation clinic during the same time period. The medical history of healthy controls revealed no signs of immune deficiency, allergic, autoimmune, or malignant diseases. Furthermore, clinical and if available also laboratory results did not show any inflammation and participants did not receive any medication affecting the immune system.

The study was accepted by the ethics committee of the University Hospital Frankfurt (#168/18), and either study subjects of legal age or custodians of underage patients agreed to participate.

Isolation of PBMCs

Ethylenediaminetetraacetic acid- (EDTA-) anticoagulated blood was collected from all subjects and stored for up to 12 h at room temperature (RT). Isolation of PBMCs was conducted by centrifugation using cell separation solution (Biochrom, Merck Millipore, Burlington, MA, USA). Therefore, EDTA-blood was diluted with Dubecco's phosphate-buffered saline (DPBS, Gibco™ Invitrogen/Thermo Fisher, Waltham, MA, USA), layered on the cell separation solution, and centrifuged for 20 min at 800*g* without break. The upper layer was transferred into another vessel, again diluted with DPBS, and centrifugated. The liquid was discarded, and the cell pellet was resuspended in DPBS. This washing step was executed twice before the cell pellet was taken up in 5 ml RPMI++ (Roswell Park Memorial Institute (RPMI, Gibco™ Invitrogen/Thermo Fisher) 1640 medium with 1% penicillin–streptomycin (Gibco™ Invitrogen/Thermo Fisher) and 10% fetal calf serum (FCS, Gibco™ Invitrogen/Thermo Fisher). The number of isolated PBMCs was determined using an Act Diff Cellcounter (Beckman Coulter, Brea, CA, USA). Subsequently, the cell suspension was again centrifugated and the cell pellet taken up in freezing solution (70% RMPI++, 10% dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO, USA), and 20% FCS) at concentrations of 1–10 × 10^6 PBMCs/ml. The PMBCs were stored at −80°C for at least 24 h using a Mr. Frosty™ freezing container (Thermo Fisher Scientific, Waltham, MA, USA) and afterward transferred into liquid N_2 for long-term storage.

Flow cytometry-based surface phenotyping

Flow cytometry analyses were used to detect lymphocyte populations and a broad repertoire of functional markers on NK and T cells. Therefore, previously frozen PBMCs were thawed in a water bath at 37.6°C and then transferred into 40-ml preheated RPMI++. After centrifugation, the cell pellet was resuspended in 1 ml RPMI ++ and the cell count as well as life–death ratio were detected using a Neubauer counting chamber (Marienfeld, Lauda Königshofen, Germany).

Subsequently, depending on blood sample volume, 0.025 × 10^6 and 0.2 × 10^6 PBMCs were washed with 1 ml DPBS. The supernatant was discarded, and the cell pellet was first resuspended in FACS buffer [Cell Wash (Becton Dickinson, Franklin Lakes, NJ, USA) + 0.5% bovine serum albumin (BSA, Sigma Aldrich) + 0.01% NaN_3 (Carl Roth GmbH, Karlsruhe, Germany)] and then incubated for 20 min with the following fluorochrome-conjugated antibodies: CD45-BV510 (clone HI30), CD3-BUV395 (clone SK7), CD56-BV421 (clone NCAM16.2), CD19-FITC (clone HIB19) (all from Becton Dickinson), CD14-BV711 (clone M5E2), and CD16-PE (clone 3G8) (all from BioLegend, San Diego, CA, USA). Afterward, the cells were washed and incubated for 5 min with 7-aminoactinomycin (7-AAD, Becton Dickinson) in FACS buffer.

Antibody-labeled cells were analyzed using a FACSCelesta™ instrument (Becton Dickinson). Data analysis was conducted using FlowJo® (Becton Dickinson).

Cell culturing for functional analyses

Based on the phenotyping results, NK cell count was adjusted to 0.1 × 10^6 or 0.2 × 10^6 NK cells/ml and cells remained either unstimulated (U) or received stimulation 1 (S1) or stimulation 2 (S2). Unstimulated cells were diluted 1:1 in RPMI++. For stimulated cells, additionally IL-2 (10^4 U/ml) (Novartis, Basel, Switzerland) and IL-15 (0.01 µg/ml) (PeproTech Inc., Thermo Fisher) were added. If possible, technical duplicates or triplicates were evaluated. Subsequently, cells were incubated for 17 h–19 h at 37°C and 5% CO_2. Then, an anti-CD107a-APC antibody (Clone H4A3, BioLegend) was added to specific conditions, followed by addition of monensin (GolgiStop™, Becton Dickinson, prediluted 1:10 with RPMI++) 1 h later to all wells. Stimulated cells received an additional boost with IL-12 (100 ng/ml) (PeproTech Inc., Thermo Fisher) and IL-18 (100 ng/ml) (MBL International Corp., JSR Micro, Leuven, Belgium) for S1 and with phorbol 12-myristat-13 acetate (PMA) (50 ng/ml) (Sigma Aldrich) and ionomycin (500 ng/ml) (PromoCell, Heidelberg, Germany) for S2. Cells were incubated at 37°C and 5% CO_2 for 2 h.

Intracellular flow cytometry analyses

After cultivation, cells were washed and resuspended in DPBS. Subsequently, the cells were incubated with 1 mg/ml

immunglobulin G (IgG, Intratect®, Biotest Pharma GmbH, Dreieich, Germany) and with 0.05 µl Zombie Violet™ (BioLegend)/µl cell suspension for 15 min at RT. After washing, cells were incubated with anti-CD56-PE (clone NCAM16.2, Becton Dickinson), anti-CD16-BV511 (clone 3G8, Becton Dickinson), and anti-CD3-PerCP (clone UCHT1, BioLegend) antibodies and incubated for 20 min at 4°C. To specific conditions, anti-FasL-APC (clone NOK-1, Becton Dickinson) or anti-TRAIL-APC (clone RIK2, BioLegend) was added.

For intracellular staining, cells were fixated with 100 µl formaldehyde 37% (AppliChem GmbH, Darmstadt, Germany) (15 min, 4°C). After washing, cells were stained in saponin buffer (DPBS + 0.2% saponin + 1% BSA) with anti-IFN-γ-FITC (clone B27, Becton Dickinson) or with anti-Granzyme B (GrzB)-FITC (clone REA22, BioLegend) antibodies and incubated for 30 min at 4°C. Lastly, cells were washed in 1 ml Perm/Wash™ buffer (Becton Dickinson) (prediluted 1:10 with distilled water) and resuspended in Perm/Wash™ buffer to be analyzed using a FACSCanto™ 10C device (Becton Dickinson). The gating strategy for the analysis of FACS data is illustrated in Supplementary Figure S2.

Statistical analysis

The joint model was used to track changes in the longitudinal trajectories of each lymphocyte subpopulation and their effect on the development of a malignancy. The longitudinal submodel was a B-spline linear mixed effect model. The Cox model fitted the time to develop a malignancy as an event taking into account death without previous cancer as a competing event and patients without any event as censored.

Univariate standardized mixed models were performed to characterize differences with age-reference models. For standardization, we took the lower limit of the 95% confidence interval of the reference values, and the standardized values were logarithmically transformed before running the mixed model to obtain an approximately Gaussian distribution for the residuals. Analyses were performed with the use of R-4.2 (R Foundation for Statistical Computing, Vienna, Austria). The nlme package version 3.1–162 and JM package version 1.5–2 were used. FACS data were analyzed using FlowJo® version 8–10 (FlowJo LLC, Ashland, ORE, USA) and analyzed using unpaired t-tests and linear mixed models with GraphPad PRISM version 6–8 (GraphPad Software, Inc., San Diego, CA, USA) as well as R-4.2. A p-value of <0.05 was considered significant.

Results

Clinical characteristics of AT patients

For this study, 65 AT patients were included of whom 57% were men and 43% were women. The median survival since birth was 27 years. There were 14 patients that developed a malignancy resulting in an incidence of 27.4% (95% CI 11.6–43.2) and 50.65% (28.7–72.6) at 20 years and at 30 years, respectively (Supplementary Figure S1). The malignant disease consisted of leukemia (n = 4), non-Hodgkin-lymphoma (n = 6), Hodgkin-lymphoma (n = 3), and meningioma (n = 1). The median age of malignancy onset was 13 years (range 1.24–29). The estimated incidence at 20 and 30 years was 7.7% (0.0–18.1) and 19.7% (1.5–37.9). Six patients died without having developed any malignant disease. The cause of death in three of these cases was respiratory failure, either due to acute infectious pneumonia (n = 1) or on the basis on chronic restrictive lung disease (n = 2). In the other three patients, causes of death are unknown based on the available patient records.

In the subgroup cohort from 12 patients (Table 1), median age at time of blood collection was 3.5 years (range 1–26) and five of the patients were wheelchair bound (ID1, ID2, ID4, ID5, ID12) as a sign for advanced neurological impairment. Only AT1 developed a malignant disease up to the timepoint of measurement—a lymphoma. Three patients received Ig-replacement therapy due to hypogammaglobulinemia, deficiency of IgG subclasses, and/or a history of frequent infections. The same patients had a medication history of adalimumab due to the development of cutaneous granulomas (ID2, ID5, ID12), whereas only in one patient (ID5) it was still administered at the timepoint of measurement. Both therapies were administered in a subcutaneous way lastly. One more patient showed cutaneous granulomas but did not receive immunomodulating therapy for this indication (ID5).

TABLE 1 Characteristics of the AT subgroup of 12 patients.

ID-Nr.	1	2	3	4	5	6	7	8	9	10	11	12
Sex	m	m	f	m	f	f	m	m	m	m	m	f
Age (years)	26	19	3	12	20	2	1	2	3	4	2	21
Malignant diseases	#1	–	–	–	–	–	–	–	–	–	–	–
Wheelchair dependence	+	+	–	+	+	–	–	–	–	–	–	+
Granuloma	–	+	–	+	+	–	–	–	–	–	–	+
Ig substitution	–	+	–	–	+	–	–	–	–	–	–	+
Adalimumab therapy	–	+			+			–	–	–	–	+

Displayed are sex, age, and history of Ig-substitution or adalimumab therapy, cancer development, wheelchair dependency, and granuloma development at timepoint of analysis. The diagnosed malignant disease in ID1 was lymphoma (#1).

In the healthy control group, 13 participants were men and 4 were women. The median age was 8 years (range 1–19).

Longitudinal immune cell analysis revealed reduced B and T cell but no difference in NK and NKT cell levels in the AT patient cohort

Longitudinal data of peripheral blood comprised 305 samples from patients who stayed cancer free and 60 of patients who developed a malignancy, but data correspond prior to the diagnosis of malignancy. The patients' age at the first measurement was in median 5.92 (range 1.09–36.86) years. Number of measurements per patient ranged from 1 to 82 (Figures 1A, B).

There was no evidence of association between the kinetics of T, NK, and B cells and the occurrence of malignancy in AT patients considering death without malignancy as competing risk event ($CD3^+$ T cells $p = 0.376$; $CD3^+CD8^+$ T cells $p = 0.541$; $CD3^+CD4^+$ T cells $p = 0.237$; $CD3^-CD56^+$ NK cells $p = 0.776$; $CD3^+CD56^+$ NKT-like cells $p = 0.321$; $CD19^+$ B cells $p = 0.725$; Figures 1C–H). One patient (light pink line) showed outstanding high numbers of T cells, which might be related to the subsequently occurring T-acute lymphoblastic leukemia (T-ALL). To avoid possible bias, the numbers of T and helper T cells of this patient were not considered in the analysis.

The longitudinal data were further compared with the corresponding age-matched reference values of healthy children and young adults (22, 23). As expected, the trajectory of absolute T cells and B cells was significantly lower in AT patients ($CD3^+$ T cells $p < 0.001$; $CD3^+CD8^+$ T cells $p < 0.001$; $CD3^+CD4^+$ T cells $p < 0.001$;

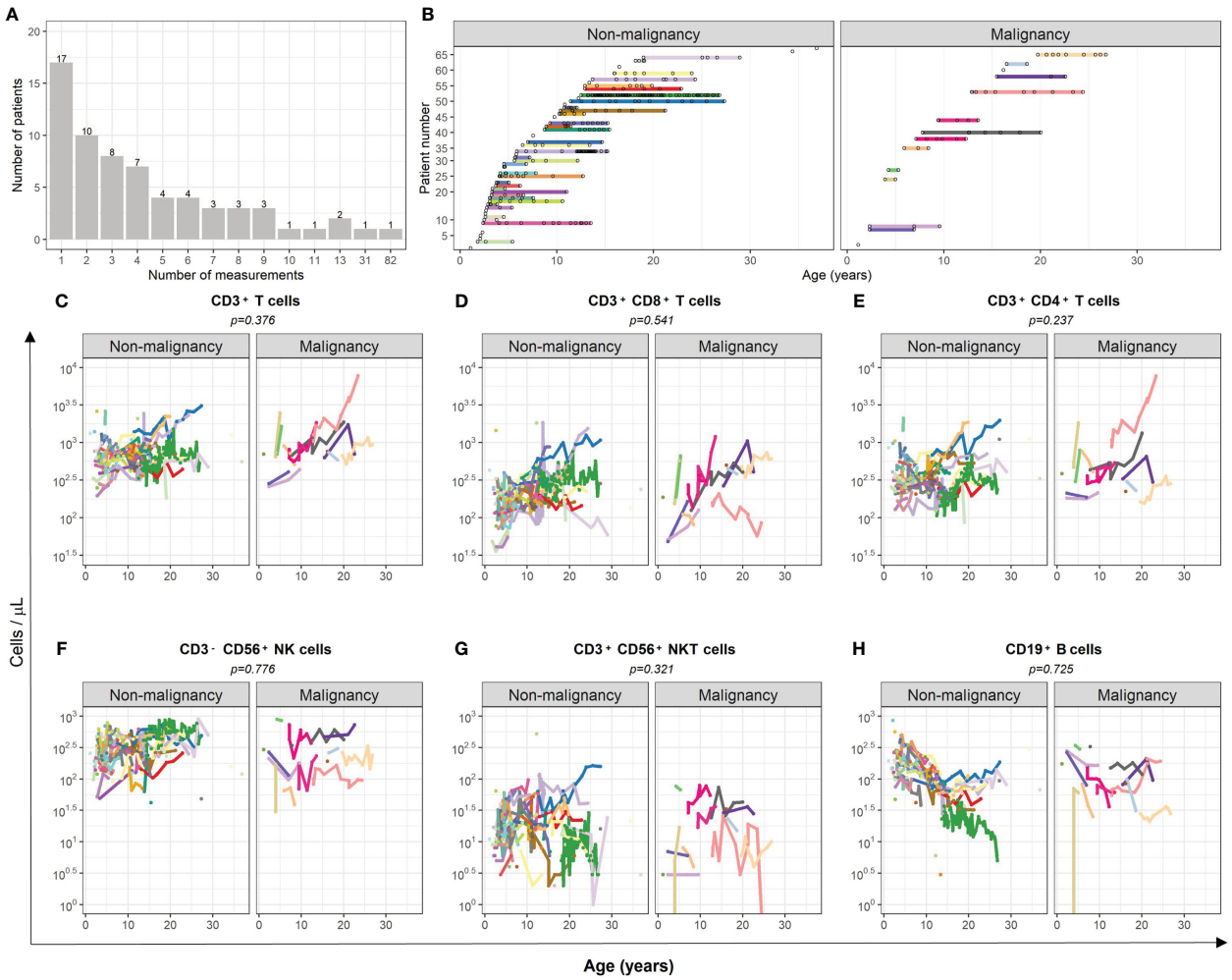

FIGURE 1
Comparison of cell numbers of different immune cell populations in peripheral blood of AT patients with and without subsequent development of malignancies. **(A)** The number of measurements of peripheral blood samples of each patient is displayed. **(B)** The time-point of each measurement of each individual patient is illustrated. **(C–H)** The total amount (cells/µl) of **(C)** $CD3^+$ T cells, **(D)** cytotoxic $CD3^+CD8^+$ T cells, **(E)** $CD3^+CD4^+$ T helper cells, **(F)** $CD3^-CD56^+$ NK cells, **(G)** $CD3^+CD56^+$ NKT-like cells, and **(H)** $CD19^+$ B cells are compared between AT patients with and without subsequent development of malignancies. Measurements after confirmation of cancer diagnosis were excluded. For statistical analysis, a mixed-effect linear model was utilized. Each model considers age and the respective cell population as covariate. Results were determined as significantly different for $p < 0.005$.

$CD19^+$ B cells $p < 0.001$; Figures 2A–C, F). However, this applies to all T-cell populations only until the age of 12 years (vertical black dashed line), whereas it applies to the B-cell population for all ages. In contrast, the NK and NKT cell counts were similar in AT patients compared with reference values (Figures 2A–F).

The subcohort of 12 AT patients displayed similar differences in absolute cell counts of immune cell populations in peripheral blood compared with healthy controls as the whole AT cohort with 65 patients (Figures 2A–F, red dots). Regarding the white blood cell distribution (WBCs), the AT subgroup displayed a significantly reduced percentage of lymphocytes and a significantly increased percentage of monocytes (Figure 2G). Within the lymphocyte population, the portions of $CD3^-CD56^+$ NK cells were highly elevated, whereas the portions of $CD19^+$ B cells and $CD3^+CD56^-$ T cells were both reduced compared with healthy controls (Figure 2H). NK cells displayed no differences in $CD16^{bright}$ and $CD16^{dim}$ subpopulations (Figure 2I).

Phenotypic and functional AT immune cell assessment indicated increased expression of death receptor ligands in stimulated NK cells together with a higher activation level of T cells after CD3-independent stimulation

With the aim of further deciphering the possible functional impairment of innate or adaptive immunity, we performed a series of functional multicolor FACS analyses to assess the activating surface markers, degranulation capacity, and IFN-γ secretion capacity of NK and T cells.

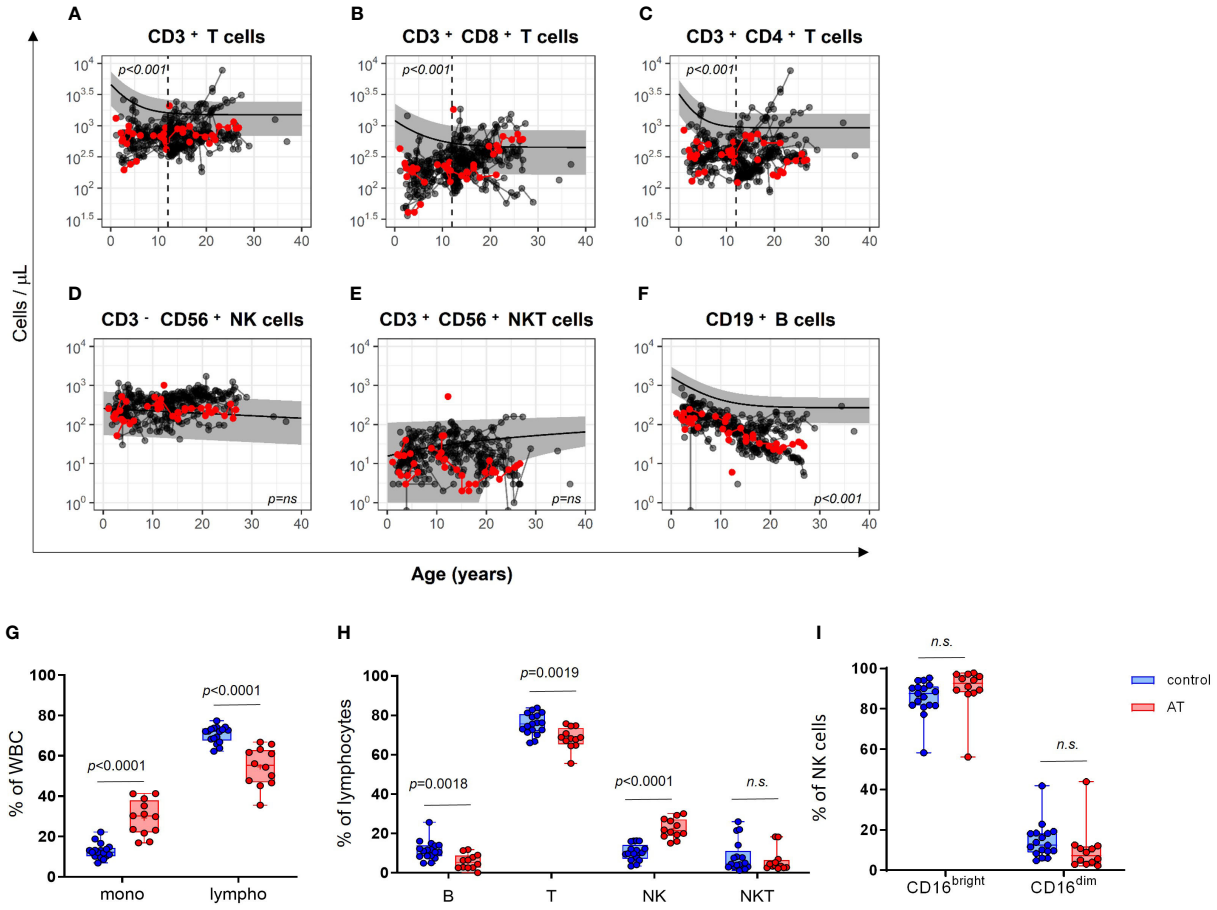

FIGURE 2
Comparison of cell numbers of different immune cell populations in peripheral blood of AT patients (n = 65) compared with healthy controls (the predicted mean reference values and its 95% confidence interval were obtained from reference model (22, 23). **(A–F)** The total amount (cells/µl) of **(A)** $CD3^+$ T cells, **(B)** cytotoxic $CD3^+CD8^+$ T cells, **(C)** $CD3^+CD4^+$ T helper cells, **(D)** $CD3^-CD56^+$ NK cells, **(E)** $CD3^+CD56^+$ NKT-like cells and, **(F)** $CD19^+$ B cells are compared longitudinally between AT patients and healthy controls. Red dots represent the 12 patients of the cohort, which was subsequently used for the analysis of functional markers. Measurements after confirmation of cancer diagnosis were excluded. For statistical analysis, linear mixed models were utilized. Each model considers age and the respective cell population as covariate. Results were determined as significantly different for $p < 0.005$. Phenotyping of white blood cells (WBC) of AT patients (AT) and healthy controls (C) analyzed with flow cytometry using isolated peripheral mononuclear cells (PBMC): **(G)** Monocytes and lymphocytes (% of WBC). **(H)** $CD19^+$ B, $CD3^+$ T, $CD3^-CD56^+$ NK, and $CD3^+CD56^+$ NKT-like cells (% of lymphocytes). **(I)** $CD16^{bright}$ and $CD16^{dim}$ subpopulations of NK cells (%). AT (red bars): n = 12, controls (blue bars): n = 17. Minimum, maximum, 25. and 75. percentiles are shown, as well as the median. For statistical analysis, unpaired t-test was used. Mono, monocytes; lympho, lymphocytes; B, B cells; T, T cells; NK, NK cells; NKT, NKT cells. n.s., not significant.

Importantly, the portion of the cytotoxically active CD16^{bright} NK cell subset was elevated in AT NK cells after stimulation with IL-12/IL-18 (p = 0.0124 (Figure 3G), which mimics a physiological stimulation by factors of the monocytic and dendritic cell components. At the same time, the IFN-γ expression seemed to be slightly reduced in this population, whereas there was no statistically significant difference compared with the control group (Figure 3A). No differences between AT patients and healthy controls were further found in unstimulated or PMA/Iono-stimulated NK cells for both IFN-γ and CD16 expression.

Simultaneously, expression levels of TRAIL and FasL were significantly higher in AT patients compared with healthy controls after stimulation of NK cells (Figures 3C, D). In particular, the expression of TRAIL after stimulation with IL-12/18 (p = 0.0044) and the expression of FasL after stimulation with PMA/Iono were increased (p = 0.0001).

Within the NK-cell subset, no significant differences in the expression of CD107a, GrzB, and perforin could be observed between AT patients and healthy controls (Figures 3B, E, F). For GrzB and perforin, however, there were notably greater variances with outstanding minimum expression levels of these proteins in the AT group.

Interestingly, regarding the T-cell functional markers, the portion of IFN-γ$^+$, CD107a$^+$, TRAIL$^+$, and FasL$^+$ cells after

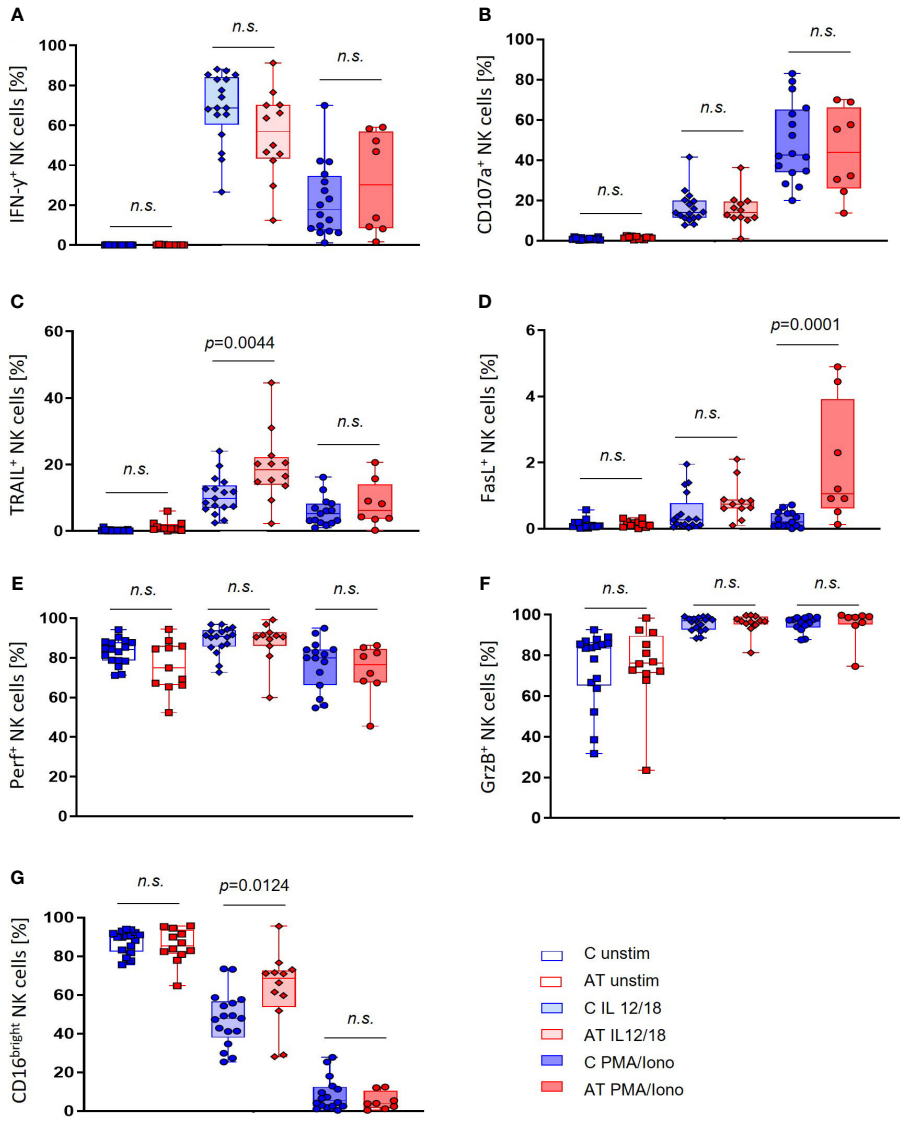

FIGURE 3
NK cell functional marker expression of AT patients (AT) and healthy controls (C) analyzed with flow cytometry using peripheral blood mononuclear cells (PBMC). Three different conditions were tested: unstimulated (unfilled bars), stimulation with IL-2/-12/-15/-18 (light filled bars), and stimulation with IL-2/-15/PMA/ionomycin (deep filled bars). **(A)** IFN-γ-expressing NK cells (%). **(B)** CD107a-expressing NK cells (%). **(C)** TRAIL-expressing NK cells (%). **(D)** FasL-expressing NK cells (%). **(E)** Perforin-expressing NK cells (%). **(F)** Granzyme B (GrzB)-expressing NK cells (%). **(G)** CD16^{bright} NK cells (%). Single values (black dots), minimum, maximum, 25. and 75. percentiles are shown, as well as the median. For statistical analysis, linear mixed models were used. AT (red bars): unstimulated n = 11–12, IL-12/-18 n = 11–12, PMA/ionomycin n = 8. Healthy controls (blue bars): unstimulated n = 17, IL-12/-18 n = 17, PMA/ionomycin n = 14–16. n.s., not significant.

stimulation with PMA/Iono was significantly increased in the AT group compared with controls (IFN-γ: p = 0.0008, CD107a: p = 0.0057, TRAIL: p = 0.0054, FasL: p = 0.0166) (Figures 4A–D). Remarkably, great variances of values within the AT group could be observed, which were not visible in healthy controls.

Concerning the GrzB and perforin expression in T cells, no significant differences between AT patients and healthy controls could be detected, even if outliers with high portions of GrzB and perforin-expressing T cells were present for each stimulation (Figures 4E, F).

Furthermore, we did not find any difference in the expression of functional markers of T cells in association with age between AT patients and healthy controls (data not shown). Concerning NK cells, however, there was a difference in the expression of FasL in association with age, with age being a positive predictor for FasL expression in AT NK cells (PMA/Iono p = 0.0326) (Figure 5).

Discussion

In patients with AT, innovative therapy concepts are urgently needed to increase quality of life and life expectancy. Detailed knowledge about the functionality of the immune system in AT patients could contribute to the establishment of novel therapies, including cellular therapy approaches. Recently, we could

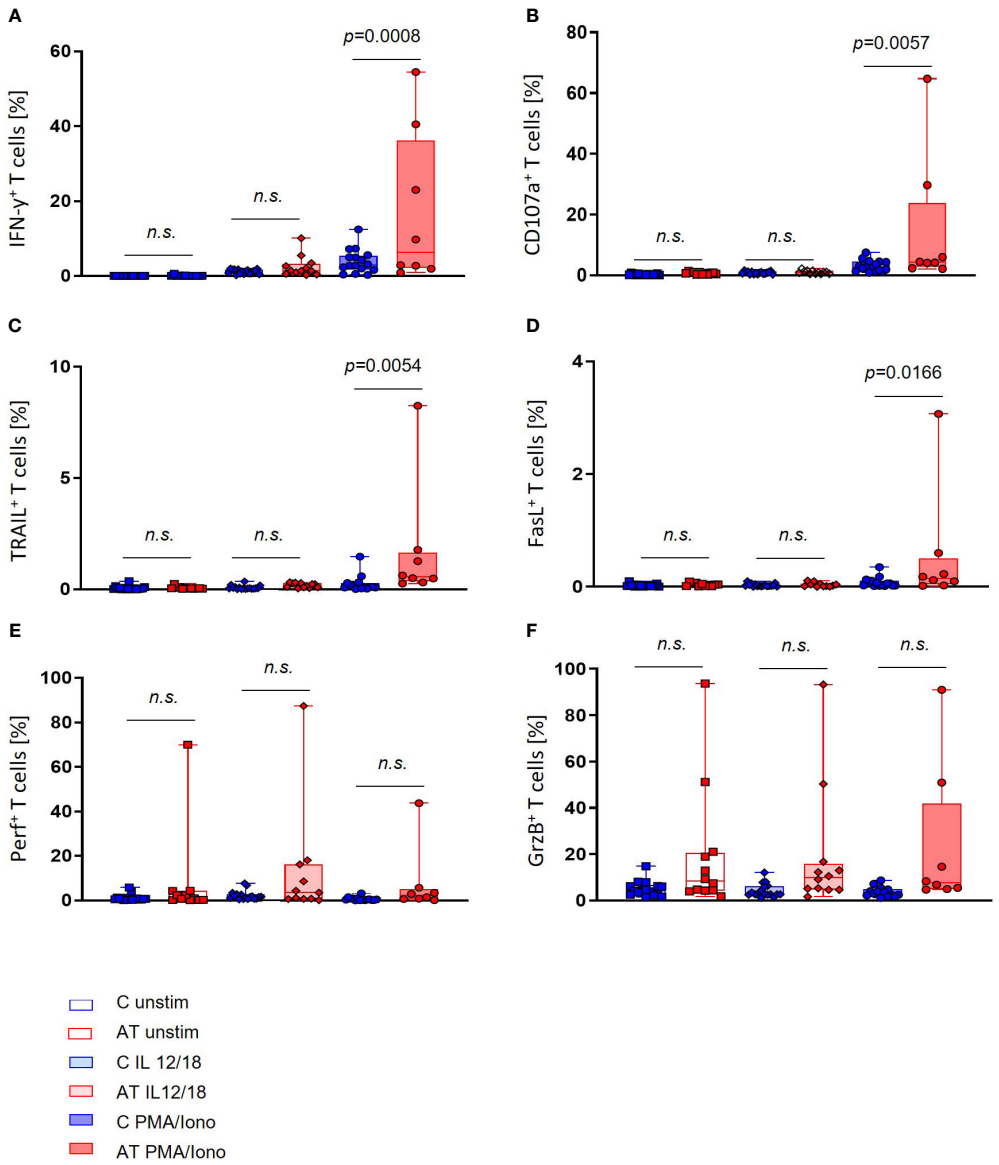

FIGURE 4
T-cell functional marker expression of AT patients (AT) and healthy controls (C) analyzed with flow cytometry using peripheral blood mononuclear cells (PBMC). Three different conditions were tested: unstimulated (unfilled bars), stimulation with IL-2/-12/-15/-18 (light filled bars), and stimulation with IL-2/-15/PMA/ionomycin (deep filled bars). **(A)** IFN-γ-expressing T cells (%). **(B)** CD107a-expressing T cells (%). **(C)** TRAIL-expressing T cells (%). **(D)** FasL-expressing T cells (%). **(E)** Perforin-expressing T cells (%). **(F)** Granzyme B (GrzB)-expressing T cells (%). Single values (black dots), minimum, maximum, 25. and 75. percentiles are shown, as well as the median. For statistical analysis, linear mixed models were used. AT (red bars): unstimulated n = 11–12, IL-12/-18 n = 11–12, PMA/ionomycin n = 8. Healthy controls (blue bars): unstimulated n = 17, IL-12/-18 n = 17, PMA/ionomycin n = 14–16. n.s., not significant.

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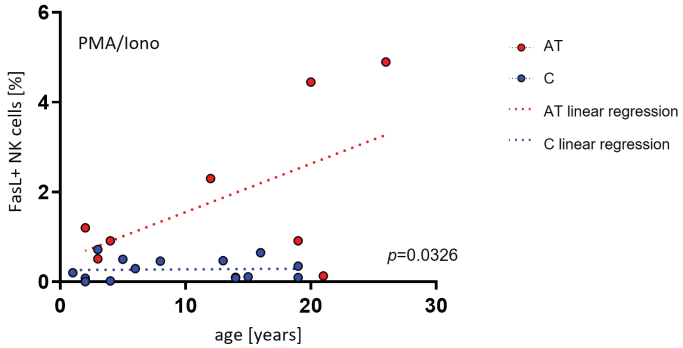

FIGURE 5
Age-dependent NK- and T-cell functional marker expression of AT patients (AT) and healthy controls (C) analyzed with flow cytometry using peripheral blood mononuclear cells (PBMC). Three different conditions were tested: unstimulated, stimulation with IL-2/-12/-15/-18, and stimulation with IL-2/15/PMA/ionomycin. Only statistically significant data are shown. FasL-expressing NK cells of AT patients and controls (%) after stimulation with PMA/ionomycin. The single values of AT patients (red dots) and healthy controls (blue dots) are shown, as well as the simple linear regression (AT = red line, controls = blue line). For statistical analysis linear mixed models were used. AT: n = 8. Healthy controls: n = 15.

demonstrate that stem cell transplantation as an individual preemptive treatment strategy is a new treatment approach from which some AT patients might benefit (25). We therefore conducted a phenotypical and functional immune cell profiling in AT patients.

Currently, we know that immunological dysfunction in AT is present in both the humoral and cellular parts of the immune system. On the cellular level, there is a reduction of B cells and of mostly naïve (CD45RA) $CD4^+$ and $CD8^+$ T cells (16). Yet, not only lymphopenia itself causes dysfunction of cellular immunity, but also an impaired stimulation of CD45RO T cells via the T cell receptor (TCR), which leads to decreased proliferation as well as IL-2 and IFN-γ production compared with healthy controls (16).

For NK cells, it is suggested that peripheral cell numbers are elevated in AT, which might be a compensatory mechanism to make up for the reduced number of T cells (16). However, also a reduced expression of NKG2D and perforin in NK cells of AT patients has recently been described, which went along with reduced NKG2D-mediated cytotoxicity (26). This indicates that alterations on a functional level could also play a role in AT NK cells.

Our analyses match these previous observations as we could also detect differences in lymphocyte subpopulations of AT patients and healthy individuals. Longitudinal data showed a significantly reduced number of both $CD4^+$ and $CD8^+$ T cells in AT patients up to the age of 12 years, as well as B cells throughout all ages compared with age-matched reference values. At the same time, however, the number of NK cells in our AT cohort was not statistically different from the reference values, so that no changes in absolute NK cell counts can be stated at this point. The fact that B and NK cells do not show age-dependent differences unlike T cells might be due to differences in cell generation and differentiation. The severe reduction in B-cell counts fits well to the observed hypogammaglobulinemia in AT patients.

Although hematological malignancies are one of the major causes of death, there was no association in the kinetics of lymphocyte subpopulations and the incidence of malignant disease in AT patients. We would therefore not suspect lymphocyte phenotype and its course to predict the evolution of malignancy in AT.

Furthermore, functional flow cytometry analyses revealed a shift in the distribution of NK-cell subpopulations toward cytotoxically more active $CD16^{bright}$ NK cells after stimulation with IL-12/IL-18, whereas the immunoregulatory $CD16^{dim}$ NK cell fraction seemed to be reduced. At the same time, the fractions of NK cells expressing the death receptor ligands TRAIL and FasL significantly differed. TRAIL expression in AT was particularly increased after stimulation with IL-12 and IL-18, and the expression of FasL was elevated after stimulation with PMA and Ionomycin. Since the death receptor-dependent cytotoxicity is involved in serial killing of pathogenic cells (27), the increased expression of these molecules in NK cells could indicate an activated NK-cell state in AT due to chronic recurrent infections.

Additionally, death receptors are involved in activation-induced cell death to maintain T-cell homeostasis (28, 29). As reconversion of CD45RO cells to CD45RA cells has been described, increased activation-induced cell death could lead to decreased reconversion to CD45RA cells. It could thereby contribute to the atypical distribution of naïve and activated T cells in AT lymphocytes (30). In this context, FasL expression on NK cells could be upregulated and induce apoptosis of activated T cells. Interestingly, the FasL expression of NK cells was highest following PMA and ionomycin stimulation, which also stimulates T cells the most. Whether the observed association between FasL expression and age in AT NK cells is thereby linked to the different kinetics in lymphocyte phenotype in AT, especially in the T-cell compartment, cannot be answered to this point and needs further evaluation.

Regarding the upregulation of CD107a in stimulated AT NK cells, however, as well as the expression of the apoptosis inducing molecules GrzB and perforin, no significant differences could be detected. We would therefore not assume function alterations of the granula-dependent killing machinery to be present in the context of

AT disease, even though the killing potential against tumor cells or the alteration of downstream elements should be studied to further elaborate on this question.

Regarding T cells, our flow cytometry analyses revealed a significantly higher expression of IFN-γ, CD107a, TRAIL, and FasL after stimulation with PMA and ionomycin in AT patients compared with healthy controls, whereas no significant differences in the fractions of GrzB- and perforin-expressing cells could be observed. It is however noticeable that for all markers, including GrzB and perforin, individual patients with outstanding high portions of expressing T cells exist, so that pronounced interindividual differences in ATM deficiency can be assumed. The extent of functional marker expression throughout age did not differ significantly between the AT and the control group.

Together, this might suggest a generally higher activation level of T cells in AT, which fits to the higher portion of CD45RO cells observed in AT. Our results are also in accordance with Schubert et al., who similarly described a higher percentage of IFN-γ^+ T cells, and with Pashankar et al., who found higher amounts of IFN-γ mRNA copies per cell in AT. At the same time, however, Schubert et al. observed an absolute decreased number of IFN-γ molecules per cell, which led to the hypothesis that T-cell activation might be on the contrary dysfunctional in AT.

Finally, it needs to be considered that an increase in the expression levels of the studied functional markers does not for itself mean an increase in functionality. A higher expression might also result from an underlying dysfunction together with compensatory mechanisms.

Furthermore, the longitudinal analysis of the AT cellular phenotype was conducted in a retrospective way and was therefore dependent on preexisting patient data. For several patients, therefore, only one measurement was available so that changes in immune phenotype over time cannot be evaluated.

Taking these limitations into account, the data presented clearly show differences in the lymphocyte phenotype of NK and T cells in AT. To evaluate how these differences translate into altered NK- and T-cell functionality, further studies are warranted.

Future studies should therefore address both the cytotoxic and regulatory potential of ATM deficient lymphocytes, as well as the phenotypic and genotypic differences in AT to meet demands of pronounced interindividual heterogeneity. A deeper comprehension of the underlying immunological dysfunctions in AT could be of importance for the development of immunotherapeutic concepts. In our opinion, novel personalized treatment strategies might be able to address different secondary diseases caused by the ATM deficiency such as the development of malignancies.

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 humans were approved by Ethics Committee of the University Hospital Frankfurt. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

LG: Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Investigation. AH: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. NA: Supervision, Visualization, Writing – original draft, Writing – review & editing. ES-M: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft, Writing – review & editing. FG: Investigation, Supervision, Writing – review & editing. SH: Resources, Writing – review & editing. CC: Resources, Writing – review & editing. SW: Resources, Writing – review & editing. HD: Resources, Writing – review & editing. JT: Resources, Writing – review & editing. T-MT: Resources, Writing – review & editing. CH: Resources, Writing – review & editing. CK: Resources, Writing – review & editing. SE: Writing – review & editing. PB: Writing – review & editing. TK: Writing – review & editing. J-HK: Writing – review & editing. SZ: Conceptualization, Resources, Writing – review & editing. RS: Conceptualization, Writing – review & editing. EU: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work has been supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG; UL 316/5–1, Project-ID 318346496 – SFB 1292) and by the Association "Help for Children with Cancer e.V." as part of the C^{3}OMBAT-AML consortium.

Acknowledgments

We thank all patients who contributed to the study by their donation of blood samples. Furthermore, we thank Prof. Alexander Steinle for the discussion of the immunological data, and Leon Buchinger for the support with the collection of patient data.

Conflict of interest

EU has no COIs directly related to this manuscript. EU has a sponsored research project with Gilead, BMS, and CRIION.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1377955/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Kaplan-Meier curves of **(A)** overall survival of the 65 AT patients since birth. OS estimate (black line) and 95% confidence intervals (shaded area) are illustrated. Median survival of the cohort was 27 years, six patients died without any malignancy. **(B)** cumulative incidence (CI) of malignancy and death without having presented a cancer. To estimate the CI of malignancy death before developing cancer was considered as competing event. The shaded area shows the respective 95% confidence intervals.

SUPPLEMENTARY FIGURE S2
Exemplary Flow Cytometry gating strategy to discriminate NK and T cells and to identify the expression of functional markers such as CD107a and IFN-γ.

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 08 August 2024
DOI 10.3389/fimmu.2024.1453046

OPEN ACCESS

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RECEIVED 25 June 2024
ACCEPTED 26 July 2024
PUBLISHED 08 August 2024

CITATION
Jiang C, He Y, Chen X, Xia F, Shi F, Xu X, Sun T and You K (2024) X-linked severe combined immunodeficiency complicated by disseminated bacillus Calmette-Guérin disease caused by a novel pathogenic mutation in exon 3 of the IL2RG gene: a case report and literature review.
Front. Immunol. 15:1453046.
doi: 10.3389/fimmu.2024.1453046

X-linked severe combined immunodeficiency complicated by disseminated bacillus Calmette-Guérin disease caused by a novel pathogenic mutation in exon 3 of the IL2RG gene: a case report and literature review

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X-linked severe combined immunodeficiency (X-SCID), caused by mutations in the gamma-chain gene of the interleukin-2 receptor (IL2RG), is a prevalent form of SCID characterized by recurrent and fatal opportunistic infections that occur early in life. The incidence of disseminated bacillus Calmette-Guérin (BCG) disease among children with SCID is much higher than in the general population. Here, we report the case of a 4-month-old male infant who presented with subcutaneous induration, fever, an unhealed BCG vaccination site, and hepatosplenomegaly. Metagenomic next-generation sequencing in blood, and the detection of gastric juice and skin nodule pus all confirmed the infection of *Mycobacterium tuberculosis*. Lymphocyte subset analysis confirmed the presence of T-B+NK immunodeficiency. Whole-exome and Sanger sequencing revealed a novel microdeletion insertion mutation (c.316_318delinsGTGAT p.Leu106ValfsTer42) in the IL2RG gene, resulting in a rare shift in the amino acid sequence of the coding protein. Consequently, the child was diagnosed with X-SCID caused by a novel mutation in IL2RG, complicated by systemic disseminated BCG disease. Despite receiving systemic anti-infection treatment and four days of hospitalization, the patient died three days after discharge. To the best of our knowledge, this specific IL2RG mutation has not been previously reported. In our systemic review, we outline the efficacy of systemic anti-tuberculosis therapy, hematopoietic stem cell transplantation, and gene therapy in children with SCID and BCG diseases caused by IL2RG gene mutation.

KEYWORDS

X-linked severe combined immunodeficiency, interleukin-2 receptor gamma-chain gene, disseminated bacillus Calmette-Guérin disease, case report, systemic review

1 Introduction

Severe combined immunodeficiency disease (SCID), a rare and fatal primary immunodeficiency disease caused by genetic defects, is characterized by a severe deficiency in T cells, B cells, and NK cells, or functional abnormalities, resulting in combined defects in humoral and cellular immunity. The incidence rate of SCID is approximately 1.5 per 100,000 live births (1, 2). Patients with SCID often develop severe opportunistic infections within 6 months of life, and without hematopoietic stem cell transplantation treatment, most die within their first year. X-linked severe combined immunodeficiency (X-SCID), caused by mutations in the interleukin-2 receptor gamma chain (IL2RG) gene, is a common type of SCID, accounting for approximately 50% to 60% of cases (3).

Disseminated bacillus Calmette-Guérin (BCG) disease is one of the most severe adverse reactions to BCG vaccination though relatively rare. The overall incidence rate of BCG disease is estimated to be 1–3.4 per 100 million persons, primarily affecting children with immunodeficiency disease and associated with high mortality (4, 5). This study reports a case of X-SCID complicated by systemic disseminated BCG disease caused by a novel mutation in IL2RG and reviews the relevant literature to summarize the clinical and genotypic characteristics of this condition. The main clinical information and genetic loci of this case are highlighted to improve the understanding of early identification and disease progression, facilitate timely initiation of immune reconstitution therapy, and assist genetic counseling efforts.

2 Case presentation

A four-month-old male was admitted to our hospital with subcutaneous induration persisting for 20 days, fever for 10 days, and poor response for 1 day. The patient was the second child of healthy, non-consanguineous parents, born full-term via cesarean section, with a birth weight of 3600 g. Two days after birth, the patient received the BCG vaccine (Chengdu Biotech, batch number: 202207a034), administered to the lower edge of the deltoid muscle in the left upper arm. At 1 week old, a hemangioma measuring approximately 5 cm was noted behind the right ear. At 3 months old, subcutaneous nodules appeared on the abdomen and later spread across the body. Subsequently, the patient developed fever, mental fatigue, poor feeding, and repeated bleeding and scabbing at the BCG vaccination site. Despite these issues, the patient's growth and development were within the normal ranges, and the patient's 6-year-old sister was healthy.

Upon hospital admission, the patient weighed 7.9 kg (P50–P75), measured 146 cm (P25–P50) in length, and exhibited a poor mental state. Scattered blue-purple subcutaneous nodules (<1 cm × 1 cm in size) were visible throughout the body. These did not fade under pressure, and some were ulcerated and scabbed (Figures 1A, B). The BCG vaccination site exhibited rupture with fluid exudation (Figure 1C). No superficial lymph node enlargement was observed, and scattered moist rales were present in both lungs. The abdomen was distended, with the liver lower edge palpable 6 cm below the right costal margin and the spleen lower edge palpable 4 cm below the left costal margin.

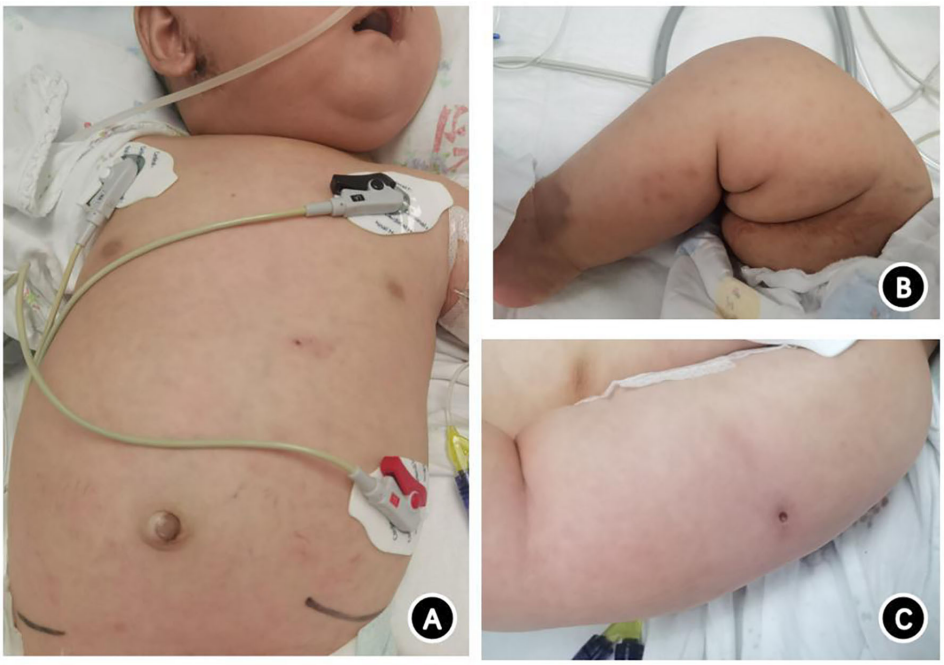

FIGURE 1
Photograph of a 4-month-old patient with SCID complicated by systemic disseminated BCG disease caused by a novel IL2RG gene mutation. **(A)** Scattered subcutaneous nodules on the trunk, with ulceration and scabbing visible below the ear and in the upper left abdomen. **(B)** Scattered rashes and purplish-blue subcutaneous nodules on the lower limbs, with some scabs forming. **(C)** Unhealed BCG vaccination site with liquid leakage.

Initial laboratory test results were as follows: white blood cell count, 2.54×109/L (4.3×109–11.3×109); lymphocyte count, 0.1×109/L (1.5×109–4.6×109); hemoglobin, 71 g/L (118–156); platelet count, 64×109/L (167×109–453×109); C-reactive protein, 154 mg/L (0–5); serum ferritin, 3737 ng/ml (13–84); D-dimer, 14234 μg/L (0–500); interleukin (IL)-6, >5000 pg/ml (0.373–0.463); procalcitonin, 6.8 ng/ml (<0.05); serum aspartate aminotransferase, 149 U/L (<30); and alanine aminotransferase, 45 U/L (<30). Immunological assessment showed an immunoglobulin G level of 0.84 g/L (7.51–15.6), immunoglobulin A level of <0.0667 g/L (0.82–4.53), and immunoglobulin M level of 0.102 g/L (0.46–3.04). Lymphocyte subset analysis revealed that the percentage of lymphocytes was 6.0% (16.8–43.4), with T, natural killer, and B lymphocytes accounting for 0.54% (55–84), 1.35% (7–36), and 93.26% (5–10) of lymphocytes, respectively. Helper and suppressor T lymphocytes accounted for 0.27% (13–41) and 0.27% (13–27) of total lymphocytes, respectively (Table 1). Acid-fast-positive bacteria were detected in the skin nodule pus puncture smear, and the proB gene test for *M. tuberculosis* in gastric juice was positive. The T cell spot test (T-SPOT) for tuberculosis (TB) infection and bacterial culture smear of cerebrospinal fluid were negative.

Chest computed tomography (CT) revealed slight inflammation in both lungs (Figures 2A, B). Abdominal CT indicated liver and spleen enlargement with some fluid accumulation in the abdominal and pelvic cavities. Bone marrow examination revealed active hyperplasia of bone marrow, neutrophils with toxic granulation and vacuoles, iron-deficient erythroid cells, and poor platelet production in megakaryocytes (Figure 2C). Langerhans cells were found on the skin print (Figure 2D) and the bovine *M. tuberculosis* complex was detected in the blood using metagenomic next-generation sequencing (mNGS) technology.

TABLE 1 Immunological data upon admission and before discharge.

Variable	Upon admission	Before discharge	Reference range
Serum immunoglobulins			
Immunoglobulin G (g/L)	0.84	-	7.51-15.6
Immunoglobulin M (g/L)	0.102	-	0.46-3.04
Immunoglobulin A (g/L)	<0.0667	-	0.82-4.53
Lymphocyte subsets			
CD3+ (%)	0.54	2.44	55–84
CD3+ (cells/μL)	1	4	690–2540
CD3+CD8+ (%)	0.27	1.33	13–41
CD3+CD8+ (cells/μL)	0	2	190–1140
CD3+CD4+ (%)	0.27	0.37	31–60
CD3+CD4+ (cells/μL)	0	1	410–1590
CD16+CD56+ (%)	1.35	0.05	7–36
CD16+CD56+ (cells/μL)	2	0	90–590
CD19+ (%)	93.26	97.13	5–10
CD19+ (cells/μL)	181	145	90–660
CD4+/CD8+	1.00	0.28	0.71–2.78

After admission, the patient was empirically treated with broad-spectrum antibiotics (Shupushen combined with linezolid) and received immunoglobulin infusion and red blood cell suspension as supportive therapy.

Lymphocyte subset analysis revealed low T and NK lymphocyte counts, alongside increased B lymphocytes, confirming T-B+NK- immunodeficiency. Therefore, genetic testing was performed on the patient, his parents, and his older sister to identify the molecular defects and genetic risk of immunodeficiency. Whole-exome and Sanger sequencing revealed a microdeletion insertion mutation in exon 3 of the IL2RG gene (chrX: 70330490–70330492), resulting in a frameshift mutation at the amino acid level of the coding protein IL2RG (NM_000206.3) gene, c.316_318 delins GTGAT p.Leu106ValfsTer42 (Figures 3A, B). This mutation was expected to cause the 106th amino acid in the protein amino acid sequence to change to valine from leucine and result in an early stop codon at the 147th amino acid, terminating translation at the 42nd amino acid and producing a truncated protein that differs from the original sequence (Figure 3C). Currently, there are no reports in the literature on this rare frameshift mutation, either domestically or internationally. Experimental data suggested that the mutation was inherited from the mother of the patients (heterozygous state), while the father did not carry this mutation. NGS of the patient's sister identified no mutation, indicating she was wild-type at the c.316_318 locus of the IL2RG gene.

The patient was ultimately diagnosed with SCID complicated by systemically disseminated BCG disease. While the attending physician recommended the patient receive systemic anti-TB treatment and immune reconstitution at a specialized hospital, the parents refused the proposal and the child was discharged on the fourth day after admission. Unfortunately, the patient died three days later.

3 Systematic review

A comprehensive search was conducted in PubMed, Web of Science, ClinVar, Embase, and Medline databases using the search terms "IL2RG," "BCG disease," and "SCID." This search yielded 11 reports and 24 children of X-SCID complicated with BCG disease caused by IL2RG gene mutation, published prior to June 2024 (Table 2). Among the 25 children (current and previous studies), all were male, with ages ranging between 1–10 months and a median age of 4.56 months at diagnosis. Most patients (n=20, 80%) presented with skin lesion symptoms, such as unhealed BCG vaccination sites, scattered rashes, subcutaneous nodules, skin rash, and eczema. Additionally, patients had pneumonia (n=22), persistent hepatosplenomegaly (n=17), persistent diarrhea (n=11),

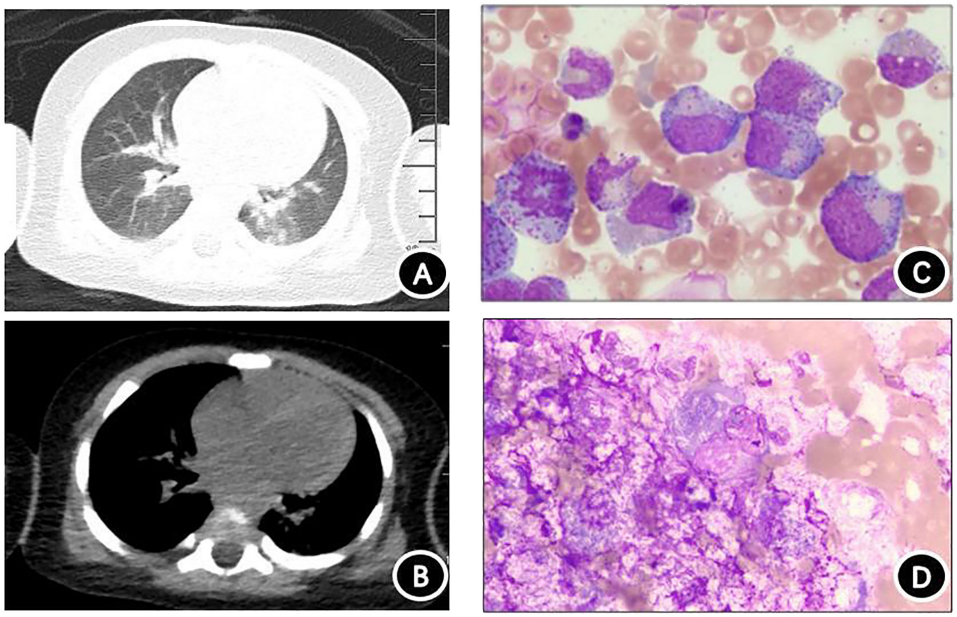

FIGURE 2
Chest computed tomography (CT) and cytological examination. **(A)** Chest CT lung window. **(B)** Chest CT mediastinal window showing scattered inflammation in both lungs. **(C)** Bone marrow specimen demonstrating significant proliferation and activation of bone marrow, with toxic particles and vacuolar degeneration visible in granulocytes. Red blood cells are mainly composed of middle- and late-stage erythrocytes, and no plate-producing megakaryocytes are present. **(D)** Rash imprint showing approximately 18% of suspected Langerhans tissue cells, with abundant cytoplasm filled with small grayish-purple particles. The nucleoli are circular or irregular in shape, and the cytoplasm is thick and loose.

sepsis (n=8), and failure to thrive (n=6) symptoms. Lymph node enlargement was observed in only one child prior to transplantation. The most commonly affected organs were the lungs, followed by the liver and the spleen.

The 23 children from whom IL2RG gene mutation sites were reported had mutations exclusively located in the coding region and all were distinct from each other. These consisted of the following mutations: exon 1 (n=2), exon 2 (n=4), exon 3 (n=7), exon 5 (n=4), exon 6 (n=3), exon 7 (n=1), and exon 8 (n=2). Only 22 children were reported about gene mutation type, 16 were point mutations (10 missense, 4 nonsense, and 2 splicing mutations), 2 were frameshift mutations caused by deletion insertion, 2 were deletion mutations, 1 contained both splicing and missense mutations, and 1 contained both deletion and frameshift mutations.

Among the 25 children, 8 received hematopoietic stem cell transplantation and 1 received gene therapy. In addition, all 9 patients received systematic anti-tuberculosis treatment (isoniazid, rifampicin, ethambutol, etc.). At the last follow-up, all 9 patients who underwent these treatments were still alive. The remaining 16 children, who did not receive immune reconstitution therapy or gene therapy, died within 1 month of diagnosis.

4 Discussion

In this report, we present a case of a 4-month-old male infant diagnosed with systemic disseminated BCG disease and X-SCID caused by a novel and rare frameshift mutation in IL2RG, which was identified using whole-exome sequencing. Moreover, we reviewed and summarized the clinical characteristics and genetic findings from previously reported cases of children with SCID caused by mutations in the IL2RG gene complicated by BCG disease.

IL2RG, located on the human X chromosome, q13.1, comprises 8 exons and 4,145 base pairs and encodes the interleukin-2 receptor gamma chain. This protein is essential for various cytokine receptors including IL-2, IL-4, IL-7, IL-15, and IL-21 (19), which regulate the differentiation and development of T lymphocytes, NK lymphocytes, and other cells. Mutations in IL2RG disrupt cytokine signaling, leading to developmental arrest of T lymphocytes, which further causes B lymphocyte dysfunction, ultimately leading to humoral and cellular immune deficiencies, namely X-SCID. In the present case, the patient exhibited clinical symptoms at 3 months of age and died within 4 weeks of onset. Similarly, all the 16 children in the literature who did not undergo immune reconstitution treatment or gene therapy died within 1 month of disease onset.

The ClinVar database catalogs over 200 pathogenic mutations in IL2RG, including missense, nonsense, frameshift, and splice site mutations, predominantly located in exons 3, 4, and 5, and mainly comprising missense mutations. In our report, we observed a novel frameshift mutation that has not yet been previously reported. As mentioned earlier, most IL2RG pathogenic mutations lead to severe T-cell defects, further causing typical SCID characterized by T-B+NK-immunodeficiency. However, atypical X-SCID can manifest as T+B+NK- immunodeficiency with milder immune deficiency

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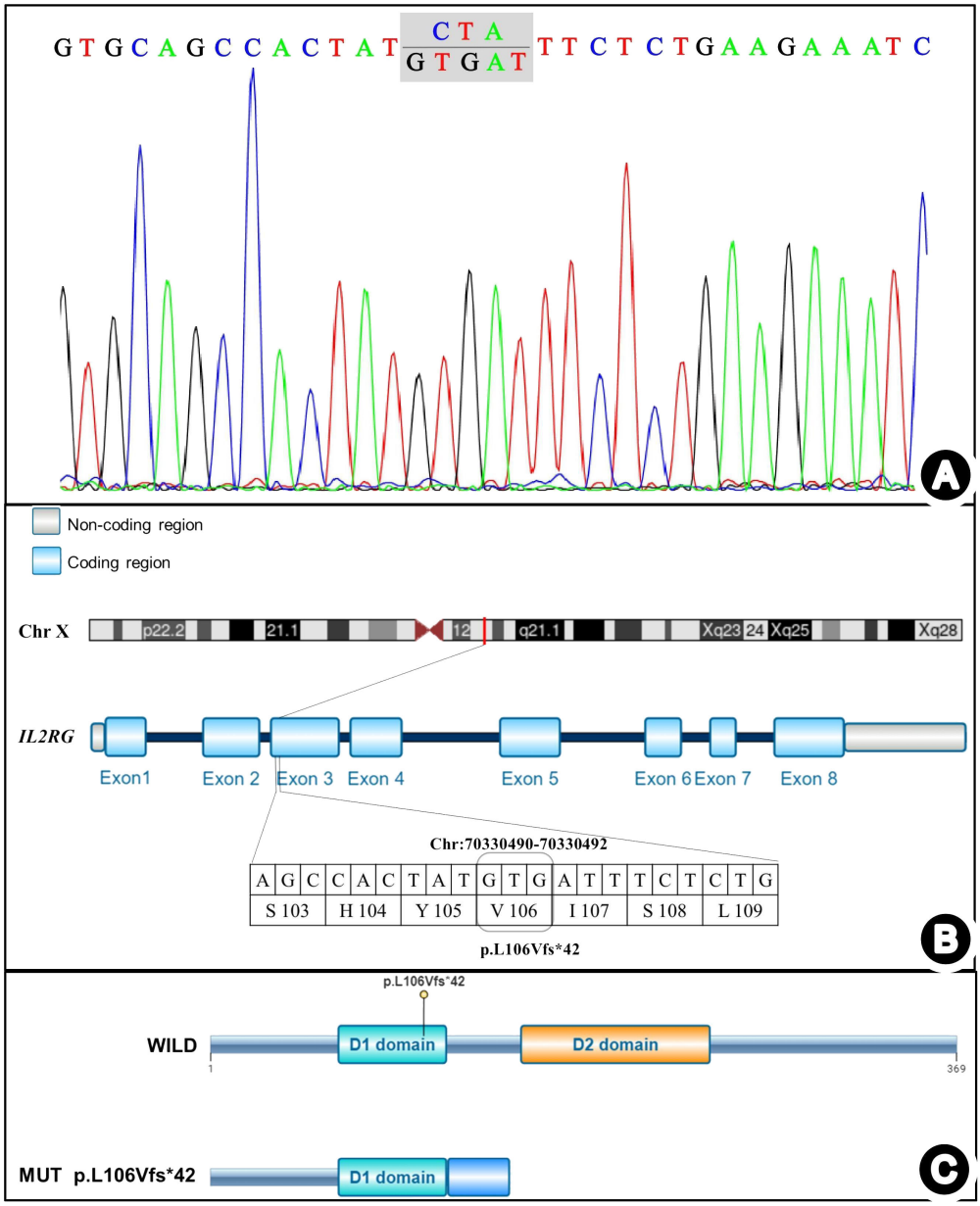

FIGURE 3
IL2RG gene mutation detected in the present case. **(A)** Sanger chromatogram. **(B)** Schematic diagram of the mutation sites. **(C)** Schematic diagram of the mutation protein.

(20). In the present case, the patient's mother was heterozygous with a normal phenotype, while the father and elder sister were wild-type, consistent with the inheritance pattern of an X-linked recessive genetic disorder. The patient exhibited T-B+NK immunodeficiency, and the onset of infection occurred between 3 and 6 months after birth, indicating that SCID caused by a rare frameshift mutation is a typical feature of X-SCID presentation. CD45Ra/Ro or detailed T-cell immune typing analysis was not conducted for the patient; therefore, we were unable to perform an in-depth assessment of the differentiation and function of immature and memory T-cell subsets, which is crucial for understanding immune dysfunction (21). According to the guidelines of the American College of Medical Genetics and Genomics, combined with the clinical phenotype and family analysis of the patient, this mutation meets the criteria for PVS1+PM2 pathogenicity classification and is considered a potential pathogenic mutation (22, 23).

SCID is prone to severe opportunistic infections caused by cytomegalovirus, Candida, Pseudomonas aeruginosa, Pneumocystis jirovecii, and BCG. BCG vaccination in children with SCID may lead to life-threatening systemic disseminated BCG disease. Studies suggest that 7% to 34% of children with SCID develop disseminated BCG disease, with the risk being 33,000 times higher than in those without SCID (5). Common manifestations of systemically disseminated BCG

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TABLE 2 Children with SCID caused by IL2RG gene mutation complicated by BCG disease.

No.	Sex	Age (months)	Clinical features	Mutation point of IL2RG gene	Gene mutation type	Nucleotide change	Treatments	Outcomes	Ref.
P1	Male	4m	Unhealed BCG vaccination site, subcutaneous induration, fever, hepatosplenomegaly, pneumonia	Exon 3	Frameshift mutation	c.316_318delinsGTGAT p.Leu106ValfsTer42	Shupushen combined with linezolid, infusion of immunoglobulin, and suspension of red blood cells.	Deceased	Present case
P2	Male	4m	Unhealed BCG vaccination site, scattered pustules, rash scabbed, fever, hepatosplenomegaly, pneumonia, dyspnea, multiple-organ dysfunction, disseminated intravascular coagulation, secondary hemophagocytic syndrome, septic shock, hepatic VOD, GVHD, subcutaneous induration	Exon 2	Deletion mutation	c.139_148delACTGACTCCC	Anti-tuberculous treatment, infusion of bone marrow and peripheral blood stem cells, defibrotide, methylprednisolone, CSA, IVIG, imipenem cilastatin, cefoperazone sulbactam sodium, methylprednisolone, ibuprofen	Symptom improvement	(6)
P3	Male	5m	Unhealed BCG vaccination site, tender swelling in the right lower forearm, hepatosplenomegaly, pneumonia, skin-colored soft nodules, cough, reactive lymphohistiocytic proliferation, granuloma	Exon 6	Nonsense mutation	c.820_823dup p.Ser275Asnfs*29	Intravenous immunoglobulin, sulfamethoxazole-trimethoprim, isoniazid, rifampin, vancomycin, piperacillin, tazobactam, azithromycin, amphotericin B liposomal, HSCT	Symptom improvement	(7)
P4	Male	4m	Hemophagocytic syndrome, fever, cough, papule, pneumonia, unhealed BCG vaccination site, dyspnea, hepatosplenomegaly, abdominal dropsy	Exon 6	Missense mutation	c.854G > A, p.Arg285Gln	CPAP, vancomycin, meropenem, IVIG, anti-tuberculous treatment, etoposide, dexamethasone	Deceased	(8)
P5	Male	8m	Fever, cough, pigmented papules, moderate oral thrush, unhealed BCG vaccination site, scattered pustules, pneumonia, massive hematemesis, melena, Pseudomonas aeruginosa sepsis	Exon 2	Missense mutation	c.136G>C	IVIG, fluid resuscitation, antibiotics	Deceased	(9)
P6	Male	5m	BCG site ulceration, pneumonia, persistent diarrhea, generalized papular rash	Exon 1	Frameshift mutation	c.8_9insA	NA	Deceased	(10)
P7	Male	6m	Recurrent pneumonia, otitis media, ulceration at BCG site, hepatosplenomegaly	Exon 8	Nonsense mutation	c.964C>T	NA	Deceased	(11)
P8	Male	4.5m	Oral thrush, recurrent pneumonia, diarrhea, failure to thrive, BCG site ulceration	Exon 1	Nonsense mutation	c.67delC	NA	Symptom improvement	(10)
P9	Male	10m	Recurrent diarrhea, pneumonia, otitis media, failure to thrive, BCG site ulceration, hepatosplenomegaly,	Exon 2	Nonsense mutation	c.202G>T	NA	Deceased	(12)

(Continued)

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TABLE 2 Continued

No.	Sex	Age (months)	Clinical features	Mutation point of IL2RG gene	Gene mutation type	Nucleotide change	Treatments	Outcomes	Ref.
			generalized adenopathy, erythroderma, eosinophilia (Omenn syndrome)						
P10	Male	6.5m	Recurrent pneumonia, BCG site ulceration, meningitis, hepatosplenomegaly, pancytopenia, transaminitis (HLH)	Exon 3	Missense mutation	c.515T>G	NA	Deceased	(10)
P11	Male	7m	Pneumonia, diarrhea, sepsis, multiple liver and spleen abscesses, oral candidiasis, hepatosplenomegaly, liver injury, anemia, skin rash	Exon 3	Disruption of RNA splicing	g.IVS3-15A>G	NA	Deceased	(13)
P12	Male	6m	Pneumonia, diarrhea, multiple liver and spleen abscesses, oral candidiasis, bone tuberculosis, hepatosplenomegaly, anemia, hypoalbuminemia, patent foramen ovale, eczema	Exon 3	Missense mutation	c.304T>C	NA	Deceased	(13)
P13	Male	4.56m	URI, pneumonia, diarrhea, hepatomegaly, failure to thrive/malnutrition, hypoalbuminemia, eczema	Exon 3	Nonsense mutation	c.312C>T	NA	Deceased	(13)
P14	Male	4.5m	Pneumonia, sepsis, urinary tract infection, oral candidiasis, oral ulcer, lymph node tuberculosis, hepatosplenomegaly, anemia, failure to thrive/ malnutrition, thrombocytopenia, renal injury, myocardial injury, pleural effusion, skin rash	Exon 3	Missense mutation	c.385T>C	NA	Deceased	(13)
P15	Male	5m	Pneumonia, diarrhea, sepsis, purulent meningitis, liver and spleen tuberculosis, CMV infection, hepatosplenomegaly, anemia, hypoalbuminemia, hemophagocytic syndrome, bilateral subdural effusion, hydropericardium, patent foramen ovale	Exon 5	Missense mutation	c.690C>T	NA	Deceased	(13)
P16	Male	5m	Diarrhea, oral candidiasis, hepatosplenomegaly, anemia, thrombocytopenia, skin rash	Exon 5	Nonsense mutation	c.738G>A	NA	Deceased	(13)
P17	Male	4m	URI, pneumonia, diarrhea, sepsis, CMV infection, hepatosplenomegaly, anemia, thrombocytopenia, hypoalbuminemia, skin rash	Exon 6	Disruption of RNA splicing/ Missense mutation	c.868G>A	NA	Deceased	(13)

(Continued)

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TABLE 2 Continued

No.	Sex	Age (months)	Clinical features	Mutation point of IL2RG gene	Gene mutation type	Nucleotide change	Treatments	Outcomes	Ref.
P18	Male	4m	Pneumonia, diarrhea, multiple liver and spleen abscesses, hepatosplenomegaly, liver injury, thrombocytopenia, hypoalbuminemia, anemia, hydropericardium, patent foramen ovale, skin rash	Exon 7	Deletion, frameshift mutation	g.IVS7-72 to IVS8-11del487	NA	Deceased	(13)
P19	Male	4m	URI, pneumonia, diarrhea, sepsis, anemia, skin rash	NA	NA	NA	NA	Deceased	(13)
P20	Male	1m	Pneumonia	Exon 5	Missense mutation	c.679T>C	Anti-tuberculous treatment and ciprofloxacin	Symptom improvement	(14)
P21	Male	1m	Pneumonia, diarrhea, sepsis, developmental delay	Exon 2	Missense mutation	c.220T>G	Anti-tuberculous treatment and ciprofloxacin	Improved	(14)
P22	Male	3m	Fever, red papules, hepatosplenomegaly, sepsis, pneumonia, coagulopathy, mild disturbance of consciousness, meningeal irritation sign, HPS	Exon 3	Missense mutation	c.391C>T; p.Gln131Ter	Nasal high-flow ventilation, anticoagulant therapy, imipenem, dexamethasone, anti-tuberculous treatment, levofloxacin, UCBT, busulfan, cyclophosphamide, immunoglobulin, etanercept	Improved	(15)
P23	Male	4m	Fever, BCG site ulceration, axillary lymphadenopathy	Exon 8	Deletion mutation	c.903_910del; p.Glu302ArgfsX110	Anti-tuberculous treatment	Improved	(16)
P24	Male	5m	Fever, cough, respiratory distress, oral thrush, bilateral pulmonary rales, hepatosplenomegaly, conjunctivitis, otitis media, pneumonia	Exon 5	Missense mutation	p.Arg226His	Trimethoprim-sulfamethoxazole, anti-tuberculous treatment, antibacterial and antifungal prophylaxis, immunoglobulin replacement therapy, BMT, treosulfan, fludarabine, ampath, linezolid, steroids	Improved	(17)
P25	Male	5m	Decreased appetite, pneumonia, hypogammaglobulinemia, tachypnea	NA	NA	NA	UCBT, broad-spectrum antibiotics, anti-tuberculous treatment	Improved	(18)

X-SCID, X-linked severe combined immunodeficiency; IL2RG, interleukin-2 receptor; BCG, bacillus Calmette-Guérin; SCID, severe combined immunodeficiency disease; T-SPOT, T cell spot test; TB, tuberculosis; CT, chest computed tomography; mNGS, metagenomic next-generation sequencing; LCH, Langerhans cell histiocytosis.
NA, Not Applicable.

include rupture and pus at the vaccination site, formation of skin masses, persistent hepatosplenomegaly, enlarged lymph nodes, and musculoskeletal pain. Due to severe lymphocyte defects in children with SCID, lymph node enlargement may not be clinically evident in patients with BCG disease, with only one child presenting with lymph node enlargement among the 25 cases included in the literature review. In the present case, the patient experienced repeated bleeding and scabbing at the BCG vaccination site and subcutaneous nodules throughout the body. The diagnosis of systemic disseminated BCG disease was based on clinical manifestation, skin nodule pus puncture smear, proB gene test in gastric juice, and mNGS, which is highly sensitive for pathogen detection (24). In cases without specific clinical manifestations or where it is difficult to detect pathogens through these conventional methods, the T-SPOT test, an interferon-γ detection test, is a TB detection method recognized by the World Health Organization (25, 26). However, the T-SOPT test in the present case

was negative, likely due to severe T lymphocyte deficiency impairing interferon-γ release. In addition, some children with SCID complicated by disseminated BCG disease may exhibit symptoms similar to Langerhans cell histiocytosis (LCH), such as body rashes, nodules, digestive issues, and bone involvement, potentially leading to misdiagnosis in the early disease stages (27). Although Langerhans cells were detected in the patient's skin print, classic LCH signs such as bone destruction were absent, ruling out an LCH diagnosis.

Systemic anti-TB therapy is essential for treating systemic disseminated BCG disease, with simultaneous immune reconstitution in patients with concurrent SCID. Currently, hematopoietic stem cell transplantation is the primary method of immune reconstitution, followed by gene therapy (28). In the literature, all 9 patients who underwent immune reconstitution therapy or gene therapy survived, while the remaining 16 children who did not receive these treatment died. Additionally, multiple studies have shown that the survival rate of children with SCID who undergo hematopoietic stem cell transplantation within 3 months after birth exceeds 90% (1, 2, 29); thus, early identification and diagnosis of SCID are crucial for improving prognosis. However, owing to the lack of characteristic clinical manifestations in the early stages, unless there is a known family history, most children with SCID can only be diagnosed after 3 months of age, delaying timely immune reconstitution. Therefore, newborn screening during the neonatal period is crucial for detecting SCID early to facilitate prompt immune reconstitution and mitigate complications from the BCG vaccination. Notably, the T cell receptor excision circle test can specifically recognize naïve T cells, which aids in screening for SCID and T lymphocyte depletion caused by other reasons (30, 31). Alternatively, the kappa-deleting recombinant excision circle test, a screening method for identifying B cell defects, can also be used for newborn screening for primary immunodeficiency (32).

5 Conclusion

We report a case of SCID complicated by BCG disease caused by a novel IL2RG gene mutation and present a literature review that outlines the benefits of immune reconstitution and gene therapy in treating patients with SCID complicated by BCG disease.

Author contributions

CJ: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing – original draft, Writing – review & editing. YH: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. KY: Funding acquisition, Resources, Supervision, Validation, Visualization, Writing – review & editing. XC: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. FX: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing. FS: Methodology, Writing – review & editing, Conceptualization. XX: Data curation, Methodology, Writing – review & editing. TS: Data curation, Investigation, Methodology, Project administration, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China Youth Fund (81501292), the China Postdoctoral Fund Project (2017M611285), and the Basic Research Program of Liaoning Province (2022JH2/101500053).

Acknowledgments

The authors thank the research group at the Department of Pediatrics of Shengjing Hospital, affiliated with China Medical University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 23 October 2024
DOI 10.3389/fimmu.2024.1495564

OPEN ACCESS

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RECEIVED 12 September 2024
ACCEPTED 08 October 2024
PUBLISHED 23 October 2024

CITATION
Frias Sartorelli de Toledo Piza C, Aranda CS, Solé D, Jolles S and Condino-Neto A (2024) Calculated globulin can be used as a screening test for antibody deficiency in children and adolescents.
Front. Immunol. 15:1495564.
doi: 10.3389/fimmu.2024.1495564

Calculated globulin can be used as a screening test for antibody deficiency in children and adolescents

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Purpose: Calculated globulin (CG, total protein minus albumin levels) correlate well with IgG levels and has been proposed as a suitable screening method for individuals with primary antibody deficiencies (PADs). We aimed to show the correlation of CG with IgG levels in children and adolescents, utilizing a common method for albumin measurement, bromocresol green.

Methods: Individuals from two Allergy and Immunology clinics were invited to participate. Inclusion criteria were age < 18, stable conditions, and signed informed consent. We included 1084 individuals. Immunoglobulin G values were determined by immunoturbidimetry; the colorimetric bromocresol green method and the Architect Biuret method were utilized for the albumin and total protein (TP) measurements, respectively.

Results: A total of 1084 individuals were included in the analysis and divided into 4 age groups (0 to <1 year, 1 to <4 years, 4 to <10 years, and 10 to <18 years). For all patients, the mean age was 6.1 (± 5) years old, the mean IgG was 9.4 (± 4.7) g/L, and CG was 23.7 (± 5.9) g/L. The most frequent diagnosis were respiratory allergies, followed by inborn errors of immunity. IgG and CG varied according to age group. Cutoff values for hypogammaglobulinemia varied from 13.8 g/L in children < 1 year to 23.1 g/L in children and adolescents aged 10 to <18 years. CG sensitivity varied from 70.9% in children aged 1 to <4 years old to 95.8% in children 4 to <10. Specificity ranged from 87.5% in children 4 to <10 years old to 100% in children and adolescents aged 10 to <18 years.

Conclusion: CG is a suitable screening test for hypogammaglobulinemia in children less than 18 years of age.

KEYWORDS

antibody deficiency, hypogammaglobulinemia, primary immunodeficiency, secondary immunodeficiency, calculated globulin (CG)

Introduction

Inborn errors of immunity (IEIs) encompass a diverse array of disorders, with over 485 unique genetic conditions and related health issues identified to date (1). Discoveries of the genetic defects underlying IEIs are occurring at an unprecedented pace. As a result, the clinical phenotypes associated with these conditions are becoming more precisely defined. This growing clarity highlights the significant health burden these diseases pose collectively. The prevalence of IEIs is currently estimated to be between 1 in 1,000 and 1 in 5,000 individuals (2).

Despite significant progress in research, including genetic sequencing and molecular diagnosis, that have enhanced our understanding of the immune system and improved the quality of life for individuals with primary immunodeficiencies (PIs), awareness of PIs remains a crucial concern for both physicians and the general public (3).

Accurate prevalence estimates of IEIs are affected by underdiagnosis, underreporting, and potential mortality before diagnosis, particularly in certain infant cases. Underdiagnosis may occur due to limited awareness, insufficient newborn screening, absence of family history or carrier testing, and asymptomatic IEIs. Patients encounter significant issues such as diagnostic delays and misdiagnosis, both of which delay appropriate treatment (4).

Primary antibody deficiency (PAD), the most frequently occurring type of IEI, is characterized by a failure to produce clinically significant levels of immunoglobulin, predominantly IgG (5). The symptoms, severity, and typical onset of PADs differ, and in addition to a heightened frequency of infections, they may also lead to complications associated with autoimmunity and malignancy (5). PADs are amenable to IgG replacement therapy (IgRT), which may prevent later organ dysfunction, reduce morbidity and mortality, and improve quality of life (6, 7).

Calculated globulin (CG, total protein minus serum albumin content) has been proposed as a reliable screening marker for early PAD diagnosis in adults (8, 9). The addition of automated calculation of CG when running total protein and albumin measurements for other conditions has been suggested (10). We have previously reported the correlation of CG, obtained from protein electrophoresis (PE) measurements, with IgG levels in children, adolescents (11), and adults (10). A significant number of patients with primary antibody deficiencies (PAD) produce seemingly adequate levels of IgG but fail to generate a protective response to pathogens, as seen in specific antibody deficiency (SAD). This group of patients may go undetected by CG screening and cannot be identified through direct IgG measurement alone. Physicians should be aware of this and consider additional testing when suspecting this condition.

There are several methods for the determination of albumin used in clinical practice. Dye-binding methods, such as bromocresol green, are the most commonly used, due to their precision and speed, compared to other methods such as PE (12).

This study aimed to assess the correlation between calculated globulin levels and serum IgG levels in children under 18 years old, using the bromocresol green method for albumin measurement. We demonstrate how this approach can aid in detecting hypogammaglobulinemia in children and lay the groundwork for future automated screening in large routine diagnostic laboratories, which is currently being initiated in Brazil.

Methods

Participant inclusion procedures

The University of São Paulo and the Federal University of São Paulo Ethics Committees approved the protocol (approval numbers 3.340.392 and 3.499.511, respectively) according to the rules and regulations of the Brazilian Ministry of Health and the Declaration of Helsinki. Patients attending two different Allergy/Immunology centers in São Paulo state, Brazil, were invited to participate, regardless of consultation purpose. The inclusion criteria included outpatients under 18 years with clinically stable conditions whose parents provided informed consent. The exclusion criteria were age above 18, unstable clinical conditions, and lack of informed consent.

Written informed consent was obtained before the inclusion of participants and blood collection.

A 5 mL blood sample was collected from each individual for laboratory analyses. Patients were free to choose the laboratory in either center. All laboratories were contacted to ascertain the measurement methods. The laboratories were accredited according to the Associação Brasileira de Normas Técnicas (ABNT NBR ISO 15189) (13) and the Brazilian Society of Clinical Pathology (PALC) (14).

Laboratory measurements

Immunoglobulin G values were determined by immunoturbidimetry (Roche COBAS 6000, Roche Diagnostics International Ltd., CH-6343, Rotkreuz, Switzerland).

The colorimetric bromocresol green (BCG) method and the Architect Biuret method (16200 Abbott Analyzer; Abbott Diagnostics) were utilized for the albumin and total protein (TP) measurements, respectively. CG values were obtained by subtracting albumin levels from the total protein values.

IgG reference values were based on Adeli et al. (15).

Statistical analysis

The assumptions of normality of the data distribution and homogeneity of variance were checked using the Shapiro–Wilk and Levene's tests, respectively. One-way ANOVA followed by Bonferroni *post hoc* correction was used to compare the IgG and CG levels between the different age groups. Simple linear regression models determined the explained variance in IgG levels based on CG levels (16, 17).

Next, receiver operating characteristic curves were constructed to identify the CG cutoff values (18) to discriminate between patients with

below-reference and normal IgG levels. Individuals with IgG values below 1.5 g/L (< 1 year), 3.2 g/L (1 to < 4 years), 5.0 g/L (4 to < 10 years), and 6.0 g/L (10 to < 18 years) were classified as below reference. The accuracy of the CG cutoff values to discriminate between patients with below-reference and normal IgG levels was verified by sensitivity (true positive rate - correct identification of patients with below-reference IgG levels) and specificity (true negative rate - correct identification of patients with normal IgG levels) tests; construction of receiver operating characteristic curves; and analysis of the areas under the curves (AUCs) and their respective 95% confidence intervals. The accuracy of the discriminant value was interpreted based on the AUC and classified as perfect (AUC = 1), exceptional (0.9 ≤ AUC < 1), excellent (0.8 ≤ AUC < 0.9), acceptable (0.7 ≤ AUC < 0.8) or poor (AUC < 0.7), considering that the AUC is not significantly different from that obtained at random for AUC values ≤ 0.5. To confirm the discriminant score, the Youden index was calculated as the highest value observed for the following operation: sensitivity + specificity – 1.

All the analyses were carried out using PASW statistics 26.0 software (SPSS Inc., Chicago, USA), with a significance level (α) of 5% (P < 0.05).

Results

A total of 1084 individuals participated in this study. IgG and CG levels increased with age, with significant differences among age groups (p < 0.001 for all comparisons) (Table 1).

The most common diagnoses were respiratory allergies (24.8%) and primary immunodeficiencies (22.1%) (see Figure 1 for other diagnoses).

Of the 249 patients with an IEI, 115 patients were receiving IgG replacement therapy (IgRT). Forty seven patients had a diagnosis of hypogammaglobulinemia, but were not receiving IgRT at the time of sample collection.

A positive and statistically significant association between IgG levels and CG levels was observed in all age groups, separately and in the combined analysis of all patients (P < 0.0001 for all, Figure 2). CG levels significantly explained part of the variance (%) of IgG levels in all age groups analyzed— < 1 year, 51% (Figure 2A); 1 to < 4 years, 60% (Figure 2B); 4 to < 10 years, 58% (Figure 2C); and 10 to < 18 years, 84% (Figure 2D)—as well as when all age groups were analyzed together (71%, Figure 2E).

For samples with below-reference or normal IgG levels, the predictive power of CG cutoff levels was classified as excellent to exceptional (AUC from 0.84 to 0.95, P < 0.01 for all), with the AUC being significant and having acceptable accuracy for all age groups (Table 2). The sensitivity and specificity ranged from 70.6% to 100%. In addition, excellent accuracy was observed for the cutoff obtained when the age groups were analyzed together (AUC = 0.86, P < 0.001, sensitivity = 77.0%, and specificity = 78.7%).

Discussion

Delays in the diagnosis and treatment of IEIs can lead to increased morbidity and mortality (19) and overall elevated treatment costs (20). Several published articles highlight that CG level can serve as an indicator of IgG levels in adults (8, 9, 21, 22), but few studies have evaluated this marker in children and adolescents.

In this report, we show that CG may serve as a screening method for children undergoing tests for other reasons. We demonstrate that cutoff values are variable, depending on the age group, from 13.8 g/L in children under 1 year of age to 23.1 g/L in children and adolescents from 10 to under 18 years of age. Sensitivity and specificity also varied according to age. In particular, the lowest sensitivity was 70.6% for samples from patients aged 1 to 4 years old, for unclear reasons.

Our group recently published a study correlating CG and IgG levels using PE for total protein and albumin measurement (11) in 1235 samples from children and adolescents less than 18 years old. The results showed that CG levels and cutoff values varied according to age group. The sensitivity varied from 75% in patients aged 2 to 3 years to 100% in patients aged 0 to 1 years and those aged 6 to 9 years, while the specificity varied from 62.7% in children younger than 1 year to 100% in adolescents aged 13 to < 18 years.

Using the bromocresol green methods for albumin measurement, Spiridonova et al. (23) evaluated 497 children (median age 8.3 years, interquartile range 2.7 to 15) for correlations between IgG and CG levels in different age groups. The best results for predicting low IgG levels were observed for a CG cutoff value of 19 g/L. The study revealed 100% sensitivity and 89% specificity for children between 0 and 17 months of age and 91% sensitivity and 90% specificity for children between 18 months of age and 2 years of age. It is difficult to compare these results with those showed in our study, since we analyzed different age groups. For the same reason, we could not compare the results with bromocresol green to our previous study using PE.

TABLE 1 Immunoglobulin G and CG levels according to age groups.

Age group	N	Mean age (years) (± SD)	Mean IgG (g/L) (± SD)	Mean CG (g/dL) (± SD)	Male sex
<1 year	147	0,49 (± 0,27)	0,27 (± 4,35)	16,79 (± 4,25)	53,7%
1 year to < 4 years	303	1,87 (± 0,82)	0,82 (± 8,36)	22,68 (± 4,24)*	50,2%
4 years to < 10 years	325	6,13 (± 1,76)	1,76 (± 10,49)	25,26 (± 4,19)*	49,8%
10 years to < 18 years	309	12,90 (± 2,30)	2,30 (± 11,82)	26,38 (± 6,53)*	46,0%
All ages	1084	6,13 (± 5,02)	5,02 (± 9,08)	23,71 (± 5,86)	49,5%

*p<0.001 when compared to last group.

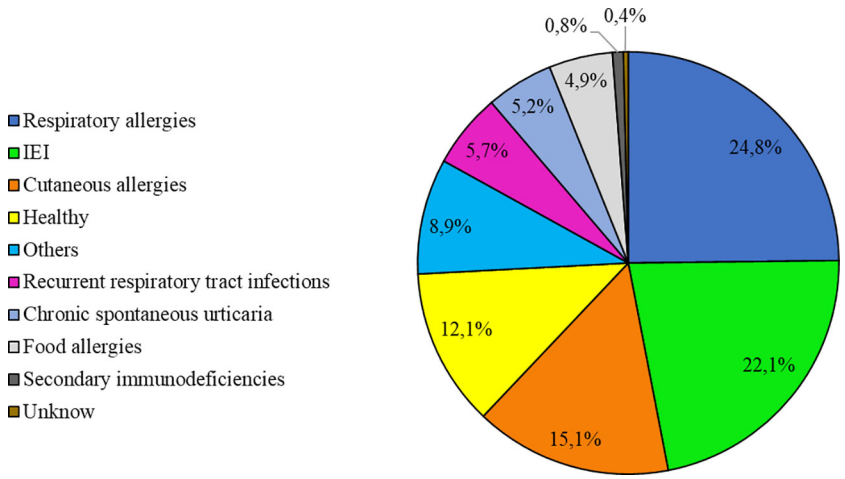

FIGURE 1
Most common diagnoses (n = 1084).

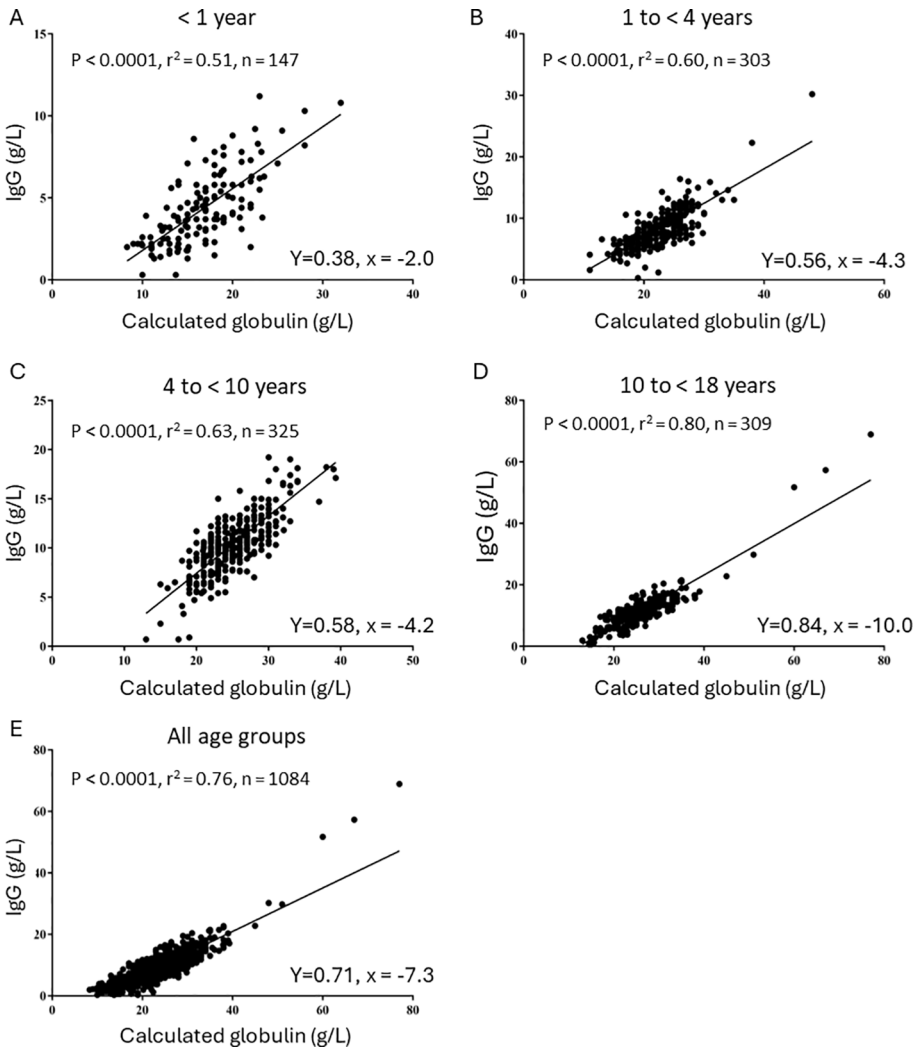

FIGURE 2
Linear regression models for the correlation between CG and IgG levels. **(A)** 0 to 1 year old. **(B)** 1 year to < 4 years. **(C)** 4 years to 10 years. **(D)** 10 to < 18 years. **(E)** All ages.

TABLE 2 AUC, 95% CI, sensitivity and especificity for CG as a screening test for hypogammaglobulinemia.

Age Group	AUC	95% CI	P value	Cut-off value (g/L)	Sensitivity	Especificity	Samples	
							Below reference*	Normal
<1 year	0.86	0.76 - 0.95	0.003	13.80	80.1%	83.3%	6	141
1 year to < 4 years	0.84	0.73 - 0.96	0.002	20.25	70.6%	85.7%	7	296
4 years to < 10 years	0.96	0.92 - 1.00	< 0,001	19.85	95.6%	87.5%	8	317
10 years to < 18 years	0.95	0.92 - 0.98	< 0,001	23.10	76.3%	100.0%	26	283
All ages	0.86	0.82 - 0.89	< 0.001	20.25	77.0%	78.7%	47	1037

*Number of samples with serum IgG values below reference for each age group.

The above studies showed that CG can be a useful screening marker for children and adolescents. Interestingly, the cutoff values for CG varied based on the age group, reflecting the different IgG levels associated with each age range.

This is the largest report of CG in children and adolescents using the bromocresol green method for albumin measurement, the most common method used worldwide. Other methods used for albumin and total protein measurements, such as our own previous study (11) using PE, are less frequently used, and in the case of PE, more labor intense.

Notably, our research results have certain limitations. The study primarily included patients who frequented allergy/immunology clinics and often had a history of repeated infections. As a result, the accuracy of the findings may differ in other demographic groups. Certain diseases may alter albumin serum concentrations (nephrotic syndrome, hepatic insufficiency etc.), and increase or decrease α and β globulins (hyperliproteineemia, metastatic malignancy, iron deficiency anemia etc.) (24). On the other hand, in infectious or inflammatory disorders, globulins may increase significantly and elevate the CG fraction, eventually yielding false negative results. In these cases, calculated globulin accuracy may be compromised. Additionally, a significant number of patients with PAD produce seemingly adequate levels of IgG but fail to generate a protective response to pathogens, as seen in SAD. This group of patients may go undetected by CG screening and cannot be identified through direct IgG measurement alone. Physicians should be aware of this condition and consider additional testing when suspecting those conditions.

If an inborn error of immunity is suspected, either because of a calculated globulin level, clinical history or other, referral to an experienced immunologist is recommended.

In conclusion, CG is a valuable screening marker for detecting hypogammaglobulinemia in individuals younger than 18. CG should not be used for patients suspected of having hypogammaglobulinemia since Ig measurements are cheaper and yield conclusive results, but rather as an opportunistic screening for patients undergoing albumin and total protein measurements for other diagnoses. We recommend that automated CG calculations be incorporated into routine practice when performing total protein and albumin measurements. This initiative is already being implemented in major diagnostic laboratories across Brazil, with the support of the Brazilian Society of Clinical Pathology, the Brazilian Association of Asthma, Allergy, and Immunology, and the Brazilian Society of Pediatrics.

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 humans were approved by The University of São Paulo and the Federal University of São Paulo Ethics Committees (approval numbers 3.340.392 and 3.499.511, respectively). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

CP: Data curation, Investigation, Methodology, Validation, Writing – original draft. CA: Investigation, Writing – review & editing. DS: Writing – review & editing. SJ: Writing – review & editing, Conceptualization. AC: Conceptualization, Writing – review & editing, Data curation, Formal analysis, Funding acquisition, Project administration, Supervision.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was funded by CSL Behring Brazil.

Acknowledgments

We thank Dr. Mariangela Correa, Conscient Scientific Consulting, for her help with manuscript writing.

Conflict of interest

AC-N declares receiving speaker's fees and participating in advisory boards for Takeda, CSL Behring, Novartis, AstraZeneca, GSK, and Sanofi Genzyme and research funding from Takeda and CSL Behring.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Case Report
PUBLISHED 20 November 2024
DOI 10.3389/fimmu.2024.1478411

OPEN ACCESS

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RECEIVED 09 August 2024
ACCEPTED 04 November 2024
PUBLISHED 20 November 2024

CITATION
Beppu S, Nishikawa T, Tomomasa D, Hijikata A, Kasabata H, Terazono H, Ikawa K, Nakamura T, Horikawa S, Nagahama J, Nakamura A, Abematsu T, Nakagawa S, Oketani K, Kanegane H and Okamoto Y (2024) Perspectives in newborn screening for SCID in Japan. Case report: newborn screening identified X-linked severe combined immunodeficiency with a novel *IL2RG* variant.
Front. Immunol. 15:1478411.
doi: 10.3389/fimmu.2024.1478411

Perspectives in newborn screening for SCID in Japan. Case report: newborn screening identified X-linked severe combined immunodeficiency with a novel *IL2RG* variant

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Background: Newborn screening (NBS) for severe combined immunodeficiency (SCID) has improved the prognosis of SCID. In Japan, NBS testing (measurement of the T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC)) was launched in 2017 and has expanded nationwide in recent years. In this study, we report a Japanese patient with X-linked SCID with a novel *IL2RG* variant identified through NBS. The patient underwent cord blood transplantation (CBT).

Case: The patient had no siblings or family history of inborn errors of immunity. He was born at 38 weeks of gestation and weighed 3,072 g. His NBS results revealed TREC 0 copies/10^5 cells (normal value: >565 copies/10^5 cells), which was considered suggestive of SCID. The patient was referred to our hospital. Although his lymphocyte count was 1,402/μL, naïve T cells and $CD56^+$ natural killer (NK) cells were decreased to 0% and 0.05% of the total lymphocytes, respectively. Flow cytometric measurement testing revealed a decrease in γc protein expression in the B lymphocytes and NK lymphocytes. We identified a hemizygous novel missense variant (c.256A>C, p.Thr86Pro) of *IL2RG*. Both *in silico* and structural analyses revealed that this variant is likely pathogenic. At 3 months of age, he underwent CBT from a human leukocyte antigen-full-

matched unrelated donor. The conditioning regimen included fludarabine (180 mg/m^2) and targeted busulfan (35 mg×h/L). The patient achieved high-level donor chimerism and immune reconstitution, including B-cell function, at 13 months.

Conclusion: Using NBS, the patient was diagnosed as having X-linked SCID with a novel missense variant of *IL2RG*. Early diagnosis using NBS tests enables safe hematopoietic stem cell transplantation without complications such as infection. We also found that even SCID with novel variants can be accurately diagnosed using the NBS program. In Japan, the test uptake rate is approximately 80% due to the high number of self-funded screening tests, and it is hoped that the uptake rate will increase in the future.

KEYWORDS

hematopoietic stem cell transplantation, *IL2RG* gene, newborn screening, structural analysis, X-linked severe combined immunodeficiency

1 Introduction

Severe combined immunodeficiency (SCID) is a rare and fatal inborn error of immunity (IEI) that is mainly caused by T- and B-cell differentiation disorders and develops in early infancy with severe infections (1, 2). Pathogenic variants have been identified in more than 20 genes that cause classical or leaky SCID phenotypes (3, 4). The most common genetic cause of SCID is variants in *IL2RG* (X-linked SCID), which encodes for the common gamma chain (γc) of the interleukin-2 (IL-2) receptor. The common γc is also shared by leukocyte receptors for other cytokines (IL-4, IL-7, IL-9, IL-15, and IL-21) that are relevant in T cell, natural killer (NK) cell, and memory B cell development; therefore, most patients have a T−B+NK−SCID phenotype (3). Hematopoietic stem cell transplantation (HSCT) is the standard curative treatment for SCID. Early HSCT before severe infection or live vaccination is an important factor for improving the prognosis of SCID (5, 6). The T-cell receptor excision circle (TREC), circular double-stranded DNA produced by rearrangement of the T-cell receptor α gene, is a molecular marker developed in 2005 that sensitively reflects normal T lymphopoiesis. In the United States, newborn screening (NBS) for SCID using the TREC quantitative polymerase chain reaction (PCR) assay proved to be successful in pilot studies starting in 2008, and by 2019, all states had contributed to an improved prognosis (7). The kappa-deleting recombination excision circle (KREC), a circular double-stranded DNA produced during B-cell receptor rearrangement, is a marker of normal B cell lymphopoiesis and was developed in 2007. In Japan, optional NBS (TREC and KREC assays) was launched in Aichi Prefecture in 2017 and is spreading to all prefectures (8). Herein, we report a case of a Japanese patient with X-linked SCID with a novel *IL2RG* variant identified through NBS.

2 Case description

Informed consent was obtained from the parents of the patient. The study was conducted in accordance with the principles of the Helsinki Declaration and the protocol was approved by the Ethics Committee on Clinical Research, Sakuragaoka Campus, Kagoshima University.

The patient had no siblings, family history of IEI, or early childhood death. He was born at 38 weeks of gestation via caesarean section and weighed 3,072 g without prenatal or delivery complications. NBS for SCID showed TREC 0 copies/10^5 cells (normal value: >565 copies/10^5 cells) and KREC 4,473 copies/10^5 cells (normal value: >456 copies/10^5 cells), which was considered suggestive of SCID. The patient was subsequently referred to our hospital for immunological evaluation. Vital signs and physical findings were unremarkable. His white blood cell count was 5,840/μL with 56% granulocytes and 24% lymphocytes (1,402/μL). The serum levels of IgG, IgA, and IgM were 846, <4, and 11 mg/dL, respectively (normal ranges: 236–1104, 10–70, and 15–110 mg/dL, respectively). In the analysis of lymphocyte subpopulations, CD3$^+$ T cells and CD56$^+$ natural killer (NK) cells were decreased to 0.2% (normal, 49–76%) (2.8/μL) and 0.05% (normal, 6.5–11.5%) (0.7/μL) of the total lymphocytes, respectively (Figure 1A), and CD19$^+$ B cells were increased to 97% (normal, 9–16%) (1,359.6/μL). The few CD3$^+$ cells were mostly memory CD8$^+$(CD45RO$^+$) cells and were considered maternal T cells (Figure 1A) because γc protein expression (CD132) was observed in the T cells (Figure 1B). In the lymphocyte stimulation test, proliferation in response to both phytohemagglutinin (PHA) stimulation and concanavalin A was low (1,171 cpm; normal value: 20,500–56,800 cpm and 1,174 cpm; normal value: 20,300–65,700 cpm, respectively). A chest radiograph revealed a thymic shadow defect. Additionally, flow cytometric measurement testing revealed a decrease in γc protein expression in B lymphocytes and NK lymphocytes

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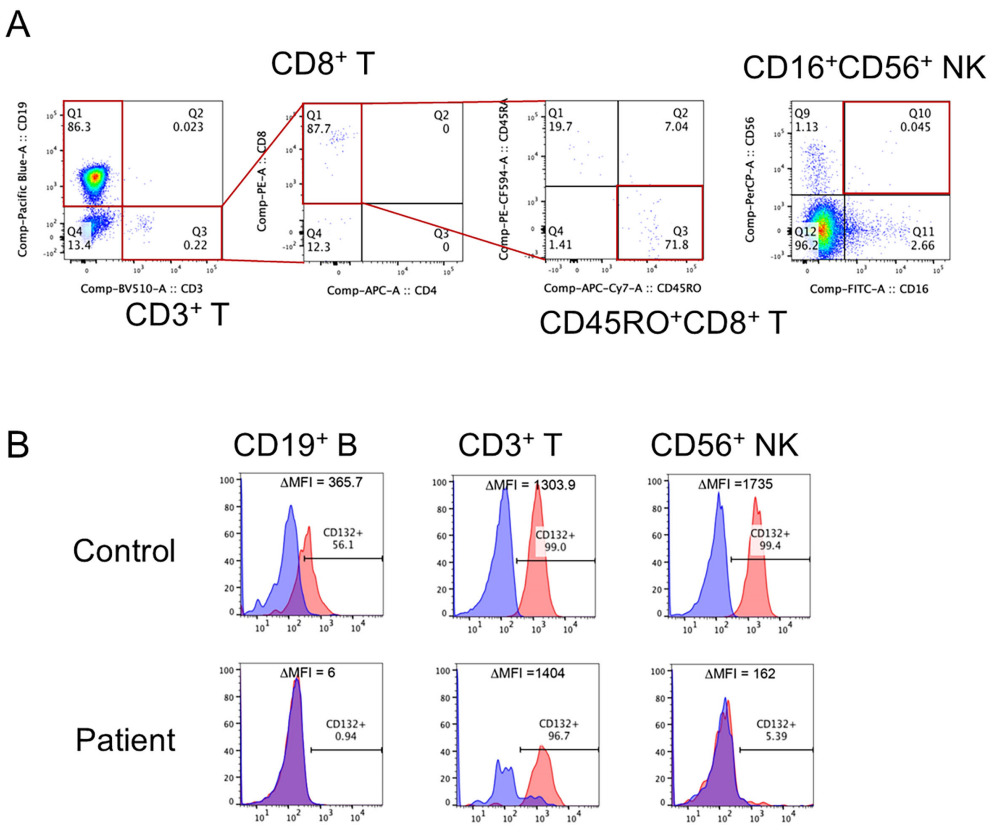

FIGURE 1
Flow cytometric analysis of lymphocyte subpopulations and expression of γc in our patient. **(A)** $CD3^+$ T cells and $CD56^+$ natural killer (NK) cells were decreased to 0.2% and 0.05% of the total lymphocytes, respectively. The $CD3^+$ T cells were mostly $CD45RO^+CD8^+$ T cells (memory $CD8^+$ T cells). **(B)** The expression of γc was lacking in $CD19^+$ B cells and $CD56^+$ NK cells.

(Figure 1B). A phenotype of T−B+NK−SCID was determined based on Primary Immune Deficiency Treatment Consortium (PIDTC) criteria and the patient was referred for HSCT evaluation (9).

2.1 Genetic analysis

Targeted panel sequencing of SCID (*IL2RG, JAK3, IL7R, RAG1, RAG2, DCLRE1C, ADA, PNP, ZAP70, LIG4, NHEJ1*, and *TBX1)* was performed at the Kazusa DNA Research Institute using genomic DNA from peripheral blood mononuclear cells. We identified a novel hemizygous missense variant (c.256A>C, p.Thr86Pro) of *IL2RG*. In the *in silico* analysis of the *IL2RG* variant, the minor allele frequency and Combined Annotation-Dependent Depletion scores of *IL2RG* were -6 and 26.2 in the c.256A>C variant, respectively (Figure 2A). In the amino acid sequence alignment of *IL2RG* among species, the amino acids in the novel variant were conserved in all species (Figure 2B).

2.2 Protein structure analysis

The atomic coordinates of the IL-2 in complex with the IL-2 receptors in humans were obtained from the Protein Data Bank (PDB) (10). The PDB contained 3D structures of IL-2 in complex with the IL-2 receptor, namely, the crystal structure of an IL-2 variant in complex with the IL-2 receptor (PDB code: 5m5e). Thr86 was located in the β-sheet of the Ig-like domain that binds IL-2; substitutions for Pro generally tend to break the β-sheet and destabilize the structure (Figure 2C). Changes in the thermal stability of the IL-2 receptor domain in response to the missense variant were evaluated using FoldX version 4 (11). The energy change of the p.Thr86Pro variant was +1.6 kcal/mol, suggesting the variant would destabilize the structure.

2.3 Patient's treatment course and follow-up

The patient was managed in a sterile room and started on a prophylactic oral sulfamethoxazole-trimethoprim combination for *Pneumocystis* pneumonia from 1 month of age. His IgG level decreased to 447 mg/dL and immunoglobulin replacement therapy was initiated at 2 months of age. There were no signs of infection, and the patient's weight increased steadily. At 3 months of age, he underwent cord blood transplantation (CBT; nucleated cell count 24.5×10^7/kg, $CD34^+$ cell count 8.9×10^5/kg) from a human leukocyte antigen-fully matched unrelated female donor.

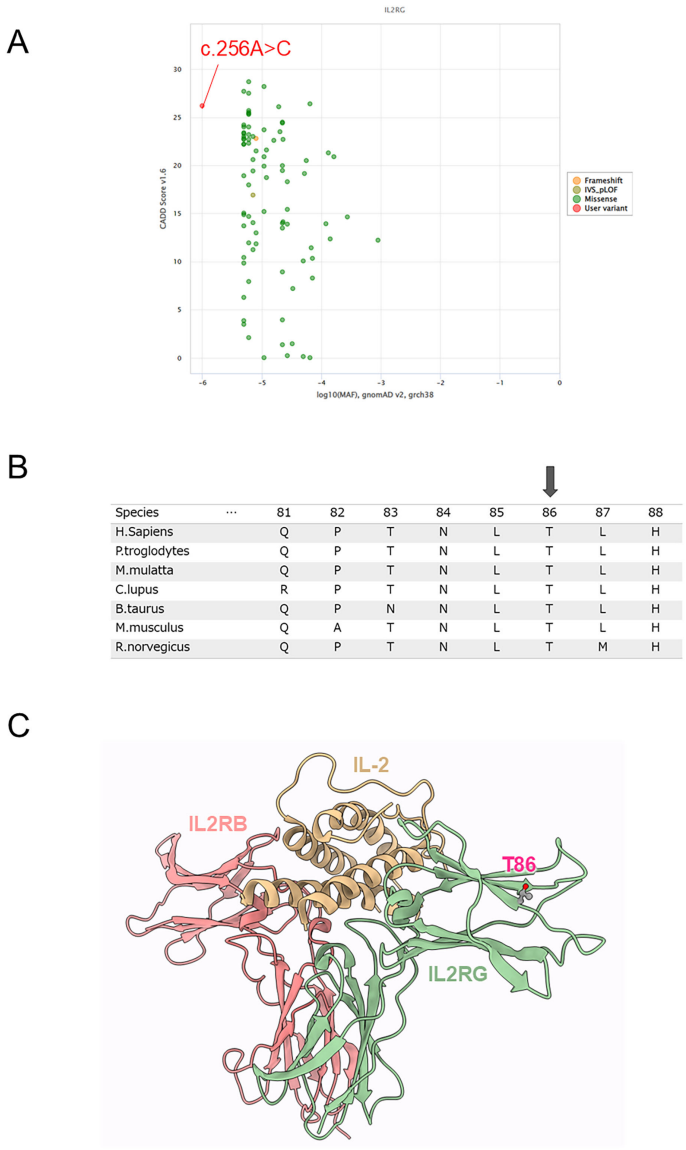

FIGURE 2
Genetic and protein structure analysis. **(A)** In the *in silico* analysis of the *IL2RG* variant, the minor allele frequency (MAF) and Combined Annotation-Dependent Depletion (CADD) scores of *IL2RG* were -6 and 26.2 in the c.256A>C variant, respectively. **(B)** Amino acid sequence alignment of *IL2RG* among species. Arrow indicates a missense variant (p.Thr86Pro). **(C)** Structural analysis of IL2RG protein. Structure of IL-2 and IL-2 receptor complex (PDB code: 5m5e). Thr86 was located in the β-sheet of the Ig-like domain that binds IL-2; substitutions for Pro generally tend to break the β-sheet and destabilize the structure.

The conditioning regimen included fludarabine (30 mg/m^2; days -7 to -2) and targeted busulfan (4 mg/kg; days -4 and -2) (total area under the blood-concentration-time curve 35 mg×h/L). Tacrolimus and short-term methotrexate were administered as prophylaxis for acute graft-versus-host disease (GVHD). Neutrophil engraftment was achieved 14 days after transplantation. High-level mixed chimerism was documented using XY chromosome fluorescein *in situ* hybridization at month 1 (81%), 3 (88%), 6 (85%), and 12 (83%). The patient's CD3$^+$, CD4$^+$, and CD19$^+$ lymphocyte counts and percentage of CD45RA$^+$CD4$^+$ T lymphocytes increased steadily, and it reached a plateau after CBT and were 927/μL, 278/μL, 2101/μL, and 16%, respectively, at 2 months after transplantation. At 1 year after the transplantation, the patient's CD3$^+$, CD4$^+$, and CD19$^+$ lymphocyte counts, percentage of CD45RA$^+$CD4$^+$ T lymphocytes, and IgG/A/M immunoglobulin level were 2,123/μL, 1,704/μL, 997/μL, 36.8%, and 726/121/238 mg/dL, respectively. Furthermore, immunoglobulin replacement therapy was not required from 2 months after transplantation to the present (13 months after transplantation).

From 73 days after CBT, the patient had a cough, and his C-reactive protein level was mildly elevated. Computed tomography revealed granular and infiltrative shadows mainly in the right lung and thickening of the bronchial wall. A comprehensive PCR test for respiratory microorganisms using a nasopharyngeal swab yielded negative results. He was diagnosed with noninfectious pulmonary complications and administered methylprednisolone from 96 days after CBT. The lung lesions and coughing rapidly improved. The steroid dose was tapered off, and the patient was discharged 126

days after CBT. Currently, 13 months after CBT, the patient is progressing without GVHD or complications, and the tacrolimus dose has been tapered off.

2.4 tSNE analysis

Peripheral blood mononuclear cells were chronologically analyzed using flow cytometry with six colors (CD45, CD3, CD16/CD56, CD4, CD19, and CD8). Standard flow cytometry files were subjected to graphics processing-unit-accelerated t-distributed stochastic neighbor embedding (tSNE-CUDA) analysis using Cytobank™ (Beckman Coulter Life Sciences, Indianapolis, IN). The tSNE-CUDA analysis allowed us to dynamically capture how each lymphocyte subset was engrafted and how it increased after CBT in our patient with X-linked SCID. $CD4^+$ cells were established first, followed by $CD8^+$, $CD56^+$, and $CD19^+$ cells, which increased in number (Figure 3).

3 Discussion

The PIDTC in the United States transplant data of 36 years and 902 patients with SCID reported a marked improvement in overall survival only in the period 2010-2018, the era of initiation and expansion of NBS (7). Additionally, the 5-year overall survival rate of patients diagnosed with SCID through NBS after 2010 is 92.5%, and they have a better prognosis than do patients diagnosed based on family history (79.9%) or clinical symptoms (85.4%) (7). Thus, the usefulness of NBS for SCID remains unequivocal.

The first NBS program in Japan, in Aichi Prefecture, started in 2017, and 137,484 newborns were screened by December 2021, with 145 (0.11%) having abnormal TREC/KREC values; two SCID cases (X-linked SCID and reticular dysgenesis) were ultimately diagnosed (8). In Kagoshima Prefecture, SCID NBS tests were started in July 2022, and 14,099 newborns were screened by March 2024, with 14 (0.1%) showing abnormal TREC/KREC values; one SCID case (present case: X-linked SCID) was finally diagnosed. In Japan, SCID NBS tests have expanded rapidly in recent years, and as of March 2024, 40 among 47 prefectures were conducting these tests (12). However, at present in Japan, most facilities require patients to pay for NBS tests for SCID. To improve the uptake rate of NBS, it is desirable for screening tests to be covered by public funds. Recently, demonstration projects have begun by national governments and, in some prefectures, the cost of the tests is being covered by public funds.

The present case was a novel missense variant of *IL2RG*; however, both *in silico* and structural analyses suggested that it was a pathological variant. Therefore, this novel variant was diagnosed as a pathological variant. After implementation of the NBS program, SCIDs of genotypes other than X-linked SCID have been diagnosed more frequently (13). Physicians should recognize this in patients diagnosed with SCID by using NBS programs. We also found that even SCID with novel variants, as in the present case, can be accurately diagnosed using the NBS program.

After SCID diagnosis, the patient was admitted to the hospital and managed in a sterile room for infection control until transplantation. Depending on the medical situation in each country, proactive approaches such as lifestyle guidance, infection surveillance, and extensive prophylaxis should be used to prevent

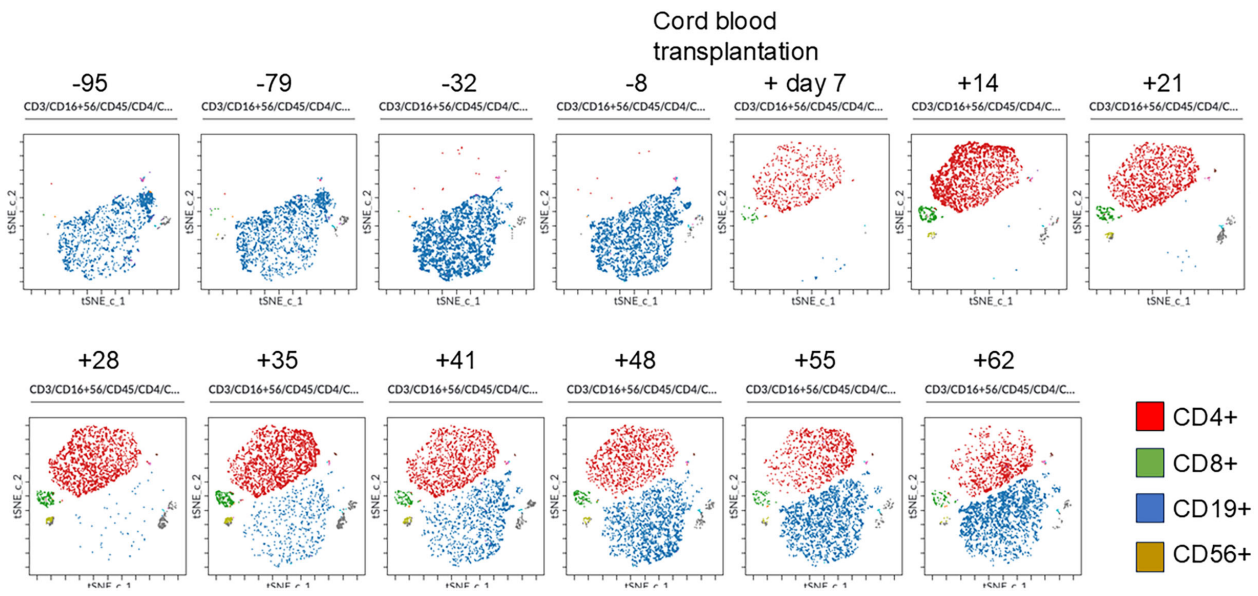

FIGURE 3
Changes in lymphocyte and natural killer cell populations by tSNE-CUDA analysis before and after cord blood transplantation. Peripheral blood mononuclear cells were chronologically analyzed using flow cytometry with six colors (CD45, CD3, CD16/CD56, CD4, CD19, and CD8). Standard flow cytometry files were subjected to graphics processing-unit-accelerated t-distributed stochastic neighbor embedding (tSNE-CUDA) analysis using Cytobank. The tSNE-CUDA analysis allowed us to dynamically capture how each lymphocyte subset was engrafted and how it increased after CBT in our patient with X-linked SCID. $CD4^+$ cells were first established, followed by $CD8^+$, $CD56^+$, and $CD19^+$ cells, which increased in number.

infection in infants until the transplantation treatment is performed (14). In a patient with SCID identified through NBS, the child appears completely normal; therefore, the family may be concerned or reluctant to have the child undergo HSCT. In our case, the medical staff understood the family's struggles and provided easy-to-understand explanations about the disease, the risks that could occur if left untreated, and HSCT. Additionally, the medical staff answered minor questions on a daily basis until HSCT, and built a relationship of trust with the patient's family. As we planned to perform HSCT using conditioning, we waited until 3 months of age to perform CBT for safety reasons. There is still no consensus regarding how long to wait for HSCT with conditioning after a SCID diagnosis through NBS. In this case, reduced intensity conditioning, fludarabine, and targeted busulfan (35 mg×h/L) were chosen because they are safe and effective strategies for obtaining high-level donor chimerism, immune reconstitution including B-cell function, and long-term survival in patients with SCID (15). We performed lineage-specific chimerism by droplet digital PCR for sex-determining region Y gene (Y-linked genes) at 2 months after CBT (16). The results showed donor-type chimerism of 90.8% in whole blood cells, 88.2% in the granulocyte fraction, 92.9% in mononuclear cells, 99.1% in T cells, and 93.2% in non-T cells (Supplementary Figure 1). CBT was performed safely with no serious adverse events, including infections. The patient developed a noninfectious pulmonary complication, which quickly resolved mildly and was otherwise unremarkable.

Using tSNE analysis, we visually and dynamically captured the respective lymphocyte subset population viability after HSCT in patients with SCID for the first time. In the present case, B-cell neoplasia was observed earlier, probably due to CBT (17). Accumulation of serial data by tSNE analysis of lymphocyte subsets of patients undergoing HSCT for SCID may predict the risk of poor engraftment and development of GVHD. Even if they appear to be in the same subset population, differences can be found using tSNE analysis.

In conclusion, we diagnosed X-linked SCID with a novel missense *IL2RG* variant using the TREC/KREC NBS test. Early diagnosis using NBS tests allowed for safe HSCT without complications, including infection. In Japan, the uptake rate of the test is 80-90% because the test is often self-funded. It is hoped that the uptake rate will be increased in the future through public funding.

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 humans were approved by Clinical Research, Sakuragaoka Campus, Kagoshima University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/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. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

SB: Writing – original draft. TNi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. DT: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. AH: Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – review & editing. HKas: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. HT: Data curation, Investigation, Validation, Writing – review & editing. KI: Investigation, Methodology, Validation, Writing – review & editing. TNa: Investigation, Writing – review & editing. SH: Investigation, Writing – review & editing. JN: Investigation, Writing – review & editing. AN: Investigation, Writing – review & editing. TA: Investigation, Writing – review & editing. SN: Investigation, Writing – review & editing. KO: Conceptualization, Investigation, Methodology, Resources, Writing – review & editing. HKan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Writing – review & editing. YO: Investigation, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was partially supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant Number #24K11003 to TNi).

Acknowledgments

We would like to thank Mrs. Maki Yamazaki for performing flow cytometry and Editage (www.editage.com) for English language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2024.1478411/full#supplementary-material

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TYPE Case Report
PUBLISHED 10 December 2024
DOI 10.3389/fimmu.2024.1498066

OPEN ACCESS

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RECEIVED 18 September 2024
ACCEPTED 20 November 2024
PUBLISHED 10 December 2024

CITATION
Chen X, Jiang C, Song W, Sun T, Yan J, Xu W and You K (2024) Case report: Identification of a Chinese patient with *RAG1* mutations initially presenting as autoimmune hemolytic anemia. *Front. Immunol.* 15:1498066. doi: 10.3389/fimmu.2024.1498066

Case report: Identification of a Chinese patient with *RAG1* mutations initially presenting as autoimmune hemolytic anemia

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Mutations in the recombination-activating gene 1, a pivotal component essential for V(D)J recombination and the formation of T- and B-cell receptors, can result in autoimmune hemolytic anemia, a rare hematological condition characterized by the autoantibody-mediated destruction of red blood cells. Herein, we report the case of a 1-year-and-4-month-old girl who presented with progressively aggravated anemia, fever, and cough. Autoimmune hemolytic anemia was confirmed by bone marrow aspiration and Coombs test. During treatment, the patient experienced two episodes of severe pneumonia and respiratory failure. Next-generation metagenomic sequencing of sputum samples confirmed the presence of cytomegalovirus and *Pneumocystis jirovecii* infections. Additionally, lymphocyte subset analysis revealed a T-B+ immunodeficiency. Whole exome and Sanger sequencing revealed a pathogenic recombinase-activating gene 1 mutation (c.2095C>T, p.Arg699Trp) and a likely pathogenic variant (c.2690G>A, p.Arg897Gln), resulting in a missense mutation in the amino acid sequence of the coding protein. Consequently, the patient was diagnosed with a recombination-activating gene 1 mutation and autoimmune hemolytic anemia as the initial presentation. This study reports a case of a recombination-activating gene 1 mutation in China and documents a combination of mutation sites and associated clinical phenotypes that were previously unreported. In this study, we outline the diverse clinical phenotypes observed in cases of recombination-activating gene 1 mutations presenting with autoimmune hemolytic anemia, aiming to facilitate timely diagnosis and appropriate treatment.

KEYWORDS

RAG1, autoimmune hemolytic anemia, V(D)J Recombination, case report, literature review

1 Introduction

The recombination-activating gene 1 (*RAG1*) is a pivotal element in V (D) J (V:Variable;D:Diversity;J:Joining) recombination (1). Mutations in this gene disrupt the normal formation of T- and B-cell antigen receptors, adversely affecting the functionality of the immune system. The incidence of *RAG1/2* gene mutations is estimated to be approximately 1.8 – 3 cases per 100,000 newborns (2–4). Mutations in *RAG1* can lead to immune dysregulation (5, 6), which can manifest as severe opportunistic infections and pose a risk of autoimmune manifestations (7, 8). Among them, autoimmune hemolytic anemia (AIHA), has been reported as a rare clinical manifestation of immune dysregulation, albeit in a limited number of cases (9). AIHA is an uncommon hematological disorder characterized by the binding of autoantibodies to the surface membrane of red blood cells, resulting in premature destruction (9). Its clinical manifestations range from mild to life-threatening anemia. Therefore, early comprehensive diagnosis, timely hemolysis control measures, and subsequent allogeneic stem cell transplantation are essential for AIHA caused by *RAG1* mutation.

Here, we present the case of a *RAG1* mutation in a Chinese patient, which initially manifested as AIHA, with an associated mutation site combination and its clinical phenotype, which have not been previously reported. Additionally, through a literature review, we have summarized the key clinical information and genotypes of all reported cases of *RAG1* mutations causing AIHA, to enhance understanding of early identification, disease progression, and timely initiation of immune reconstitution therapy, as well as to support genetic counseling.

2 Case description

A 1-year-and-4-month-old female, born full-term via cesarean section and weighing 3860 g at birth, is the first child of unrelated, healthy parents. She had previously been in good health; however, on May 5, 2024, she was admitted to the hospital with a persistent fever and progressively worsening anemia. Upon admission, a decline in hemoglobin levels was observed, accompanied by an elevation in reticulocyte count and percentage. A positive Coombs test and bone marrow cytology findings confirmed the diagnosis of AIHA (Supplementary Figure 1). After treatment with hormones, intravenous immunoglobulins (IVIG), intermittent low-volume blood transfusions, and plasmapheresis, the patient's anemia and hemolytic symptoms gradually improved. Nonetheless, transient decreases in white blood cell and granulocyte counts were observed. During treatment, the patient developed a cough and progressively aggravated dyspnea. Targeted next-generation sequencing (tNGS) of sputum samples confirmed the presence of cytomegalovirus (*CMV*), while chest computed tomography (CT) scans revealed associated abnormalities or lesions (Figures 1A, B).

The patient was administered ventilator-assisted ventilation and ganciclovir as *CMV* antiviral therapy. Mechanical ventilation was discontinued on day six of admission. By the 18th day of hospitalization, the patient's symptoms had gradually resolved, allowing for discharge. After discharge, the patient was prescribed intermittent low-dose glucocorticoid therapy. One month later, follow-up chest CT at the outpatient clinic indicated significant improvement in the bilateral lung inflammation (Figures 1C, D). Routine blood re-examination revealed no signs of hemolysis.

However, two months after discharge, the patient was admitted to hospital on July 31, 2024, due to dyspnea accompanied by retraction and cyanosis of the upper extremities. The patient was conscious but in poor general condition, exhibiting tachypnea, nasal flaring, and three positive concave signs. Immediately upon admission, the patient was intubated and provided ventilator-assisted ventilation. The tNGS of sputum revealed *Pneumocystis jirovecii* infection, and the chest CT showed findings shown in Figures 1E, F. Immunological assessment showed an immunoglobulin G level of 6.17 g/L (7.51–15.6), immunoglobulin A level of 0.2 g/L (0.82–4.53), and immunoglobulin M level of 0.51 g/L (0.46–3.04). Lymphocyte subset analysis revealed that T, natural killer, and B lymphocytes accounted for 17.19% (55–84), 35.18% (7–36), and 44.18% (5–10) of lymphocytes, respectively. Helper and suppressor T lymphocytes accounted for 0.45% (31–60) and 5.19% (13–41) of the total lymphocytes, respectively (Table 1). These results confirmed the possibility of T-B+ immunodeficiency. Therefore, the patient was treated with trimethoprim-sulfamethoxazole, and the dyspnea gradually improved. In both hospitalizations of the patient, the patient's inflammatory markers were continuously monitored, and the specific trends and values are presented in Supplementary Figure 2.

Given the patient's history of autosomal hypopomorphic immunodeficiency associated with AIHA, and past infections with *CMV* and *Pneumocystis jirovecii*, we performed genetic testing of the patient and her parents to identify the molecular and genetic risk of immunodeficiency (Figure 2). Whole-exome and Sanger sequencing identified a substitution mutation (c.2095C>T) in exon 2 of *RAG1* (chr11:36596949), leading to a missense mutation at the amino acid level in the encoded *RAG1* protein (NM_000448.3). This mutation results in substituting the 699th amino acid, arginine (Arg), with tryptophan, denoted as p.Arg699Trp, which is classified as a pathogenic variant. This mutation was classified as a pathogenic variant, is rare with a gnomAD frequency of 0.0000080, and reported as deleterious by the Combined Annotation Dependent Depletion (CADD) score of 22.8. Experimental data indicated that the patient carried the mutation in a heterozygous state, specifically inheriting it from her mother, while the father had a wild-type at this locus. Furthermore, we identified a likely pathogenic variant [NM_000448.3(*RAG1*):c.2690(exon2)G>A; p.(Arg897Gln)], in which a substitution mutation occurred at nucleotide position 2690 within the coding sequence of the *RAG1* gene. This led to a missense mutation, causing the 897th amino acid to be replaced with glutamine (Gln). Figure 2 presents the two components: 1) the Sanger chromatogram depicts both pathogenic and likely pathogenic variants; 2) a three-dimensional structural diagram of the protein post-mutation showcase the pathogenic and likely pathogenic variants of *RAG1*, which were generated using PyMOL 2.1.0 (PyMol Molecular Graphics System, Schrödinger, LLC). This study reports a case of a recombination-activating gene 1

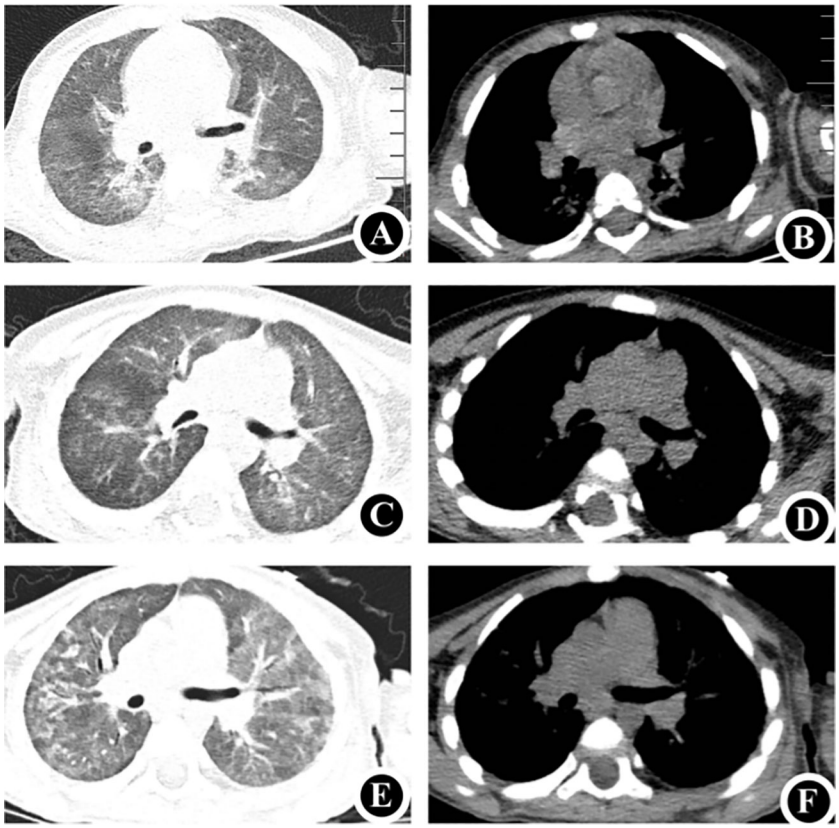

FIGURE 1
Chest CT **(A, B)** May 5, 2024: Demonstrates uneven bilateral lung transparency with multiple ground-glass opacity (GGO) patchy shadows, consistent with the manifestations of *CMV*. **(C, D)** June 5, 2024: Compared to the previous scans, a noticeable decrease was observed in the extent of GGO patchy shadows in both lungs. **(E, F)** August 5, 2024: Reveals a decrease in bilateral lung field transparency and an increase in multiple GGO patchy shadows, consistent with the typical manifestations of *Pneumocystis jirovecii* pneumonia. CT, computed tomography.

mutation in China and documents a combination of mutation sites and associated clinical phenotypes that have not been reported previously.

The patient had received Bacillus Calmette-Guérin (BCG) vaccine at birth. During the observation period of this study, the patient did not exhibit symptoms of BCG disease nor signs of tuberculosis infection. Up to the final follow-up, the patient continued to receive IVIG replacement therapy and antibiotic prophylaxis.

3 Systematic review

A comprehensive search was undertaken across the Web of Science, ClinVar, Embase, and Medline databases, using the search terms "*RAG* " and "hemolytic". In June 2024, this study compiled and published findings from 13 reports encompassing 41 children diagnosed with AIHA resulting from mutations in the *RAG 1* gene (Table 2). Among the 41 children (included in current and prior studies), 22 (54%) were male. With the exception of one patient whose age remained unascertained, the median age at diagnosis was 3.75 years (range, 1 month – 17 years).

The clinical phenotype of *RAG* mutations manifested primarily as delayed combined immunodeficiency, characterized by granuloma or autoimmunity, known as CID-G/AI (n=18, 44%), followed by leaky/atypical severe combined immunodeficiency (SCID) (39%), and SCID (n=4, 10%), and Omenn syndrome (n=3, 7%). A breakdown of autoimmune complications revealed that 17 patients exhibited simple AIHA, eight had AIHA concomitant with immune thrombocytopenia (ITP), eight presented with AIHA and autoimmune neutropenia (AN), and six had AIHA in combination with AN and ITP. Eight children displayed granulomatous manifestations, seven of whom had cutaneous granulomas, and two presented with extracutaneous manifestations (affecting the lungs, liver, bone, pancreas, and testes in one case, and bone in another). Notably, one child manifested granulomatous changes exclusively in the lungs. Among other clinical symptoms, pneumonia (including a case of typical *Pneumocystis jirovecii* pneumonia in our patient) was reported in six patients, accompanied by various skin alterations (vitiligo, dermatitis, urticaria, chickenpox, alopecia, and scabies) in five patients, digestive disorders (enteritis, diarrhea, and ptosis) in four patients, urinary diseases (nephritis and nephropathy) in two patients, and neurological conditions (Guillain-Barré syndrome and demyelinating diseases) in four patients. Hepatosplenomegaly was identified in five patients. *CMV* (N=5), herpes virus (N=3), bacteria (N=3), and isolated cases of RSV, rotavirus, parasitic, tuberculosis, and fungi were also reported.

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TABLE 1 Immunological data upon admission and before discharge.

Variable	Admission in May, 2024	Admission in July, 2024	Reference range
Serum immunoglobulins			
Immunoglobulin G (g/L)	10.23	6.17	7.51–15.6
Immunoglobulin M (g/L)	3.72	0.51	0.46–3.04
Immunoglobulin A (g/L)	0.4	0.2	0.82–4.53
Lymphocyte subsets			
CD3+ (%)	5.80	17.19	55–84
CD3+ (cells/μL)	84	73	690–2540
CD3+CD8+ (%)	5.78	5.19	13–41
CD3+CD8+ (cells/μL)	38	22	190–1140
CD3+CD4+ (%)	1.17	0.45	31–60
CD3+CD4+ (cells/μL)	8	2	410–1590
CD16+CD56+ (%)	35.34	35.18	7–36
CD16+CD56+ (cells/μL)	233	150	90–590
CD19+ (%)	48.93	44.18	5–10
CD19+ (cells/μL)	323	189	90–660
CD4+/CD8+	0.20	0.09	0.71–2.78

All 41 patients (100%) exhibited positive manifestations of Coombs' autoantibody, along with other positive antibodies including anti-platelet antibodies (N=6), anti-neutrophil antibodies (N=5), anti-TPO antibodies (N=4), anti-IFN-α autoantibodies (N=3), and anti-nuclear antibodies (N=1).

Twenty-five patients were treated with IVIGs, 23 of these received treatment before hematopoietic stem cell transplantation (HSCT). Twelve patients were treated with rituximab, four of whom underwent plasma exchange. Twenty-eight patients who underwent HSCT had a median age at transplantation of 3.7 years (range, 6 months – 20 years). Of 11 patients that died, eight succumbed after HSCT and three before transplantation.

4 Discussion

In this report, we present a case of missense mutation in the *RAG1* gene causing AIHA as the first symptom, followed by *CMV* infection and severe *Pneumocystis jirovecii* pneumonia in a 1-year and 4-month-old female in China. The mutations were identified using a combination of whole-exome and Sanger sequencing. Additionally, we reviewed and summarized the clinical manifestations and genetic results of previously reported cases of AIHA associated with *RAG1* mutations.

RAG comprises a catalytic subunit, *RAG1*, and an essential cofactor, *RAG2*. The core of *RAG1* contains residues of the active site responsible for DNA cleavage and establishes extensive sequence-specific and non-specific interactions with RSS (Recombination Signal Sequence) and flanking DNA (24, 25). In combination with *RAG2, RAG1* forms a potent genome editing tool for lymphocytes by initiating V (D) J (V:Variable;D:Diversity;J:

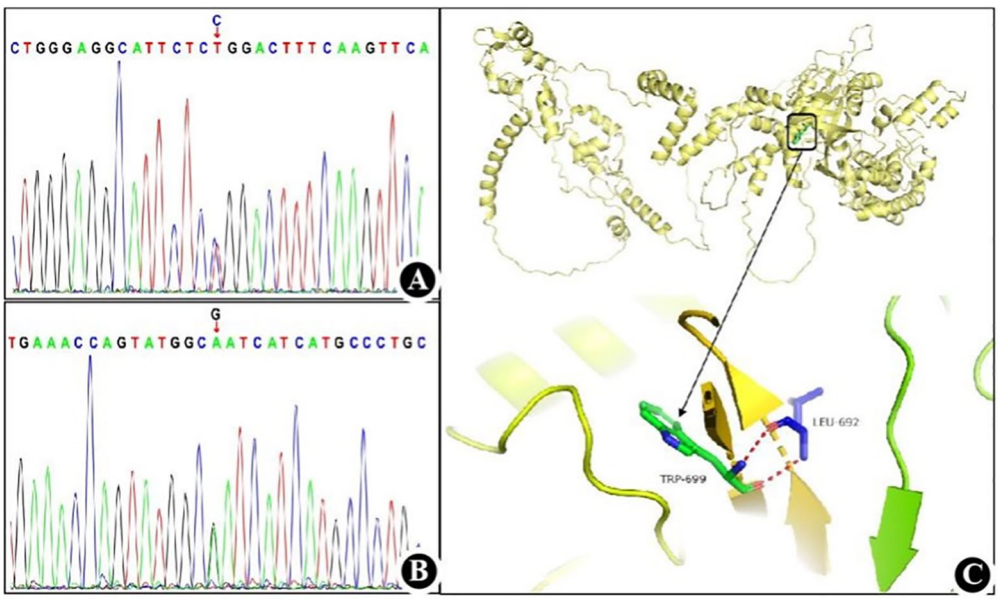

FIGURE 2
RAG1 mutation detected in the present case **(A)** Sanger chromatogram of the pathogenic variant. **(B)** Sanger chromatogram of the likely pathogenic variant. **(C)** Three-dimensional structure diagram of protein after *RAG1* pathogenic mutation.

Joining) recombination (26). Polyclonal libraries of functional T and B lymphocytes expressing a diverse array of productive T cell receptor and B cell receptor rearrangement libraries were previously generated (26, 27). These libraries were strictly controlled to prevent dysregulation (28). However, in cases of dysregulation, ineffective mutations in *RAG1* and *RAG2* led to the SCID phenotype (29), as defined by the Primary Immune Deficiency Treatment Consortium in 2022 (30). Nevertheless, hypomorphic *RAG* mutations are associated with a range of clinical and immunophenotypes, including Omenn syndrome (24, 29, 30), leaky SCID and atypical SCID, which do not display typical Omenn syndrome characteristics, and SCID (31). Patients presented with a total T cell percentage of merely 0.5% (normal range: 55%–84%), and < 20% of CD4+ T cells were naive; these patients have been classified as exhibiting leaky/atypical SCID clinical phenotypes. The marked reduction in the total T cell percentage may be linked to impaired central T cell tolerance, subsequently facilitating the development of an anti-cytokine antibody restriction mode (30–33). The main clinical manifestations included AIHA and recurrent pneumonia, which were mainly caused by *CMV* and *Pneumocystis jirovecii*.

The ClinVar and dbSNP databases have documented over 300 mutation types in *RAG1*, including nonsense, frameshift, intra-frame deletions or insertions, and missense variants in *RAG1* and *RAG2*, which affect various protein domains. The two mutations we reported were located in exon 2 of *RAG1* [NM_000448.3(*RAG1*): c.2095C>T, p.(Arg699Trp)] and exon 2 of *RAG1* [NM_000448.3(*RAG1*): c.2690G>A, p.(Arg897Gln)]. While these mutations have been reported in gene banks, the clinical phenotype caused by these two mutations is still unclear. Regarding the first missense mutation, the patient's mother was heterozygous and exhibited a phenotypic manifestation, whereas the father was wild-type. Whereas, the patient's mother was wild type and phenotypically normal for the second genetic missense mutation, but the father was heterozygous, adhering to the genetic pattern of autosomal recessive inheritance, specifically compound heterozygous inheritance. According to the genetic test results, this condition was attributed to a rare missense mutation in *RAG1*. Following the guidelines established by the American College of Medical Genetics and Genomics and based on the clinical phenotype and familial analysis of patients, mutation 1 was deemed a pathogenic mutation, whereas mutation 2 was classified as a possible pathogenic mutation. However, substantial evidence exists for mutation 2, pointing towards its eventual classification as pathogenic, although it is currently insufficient to establish its pathogenicity definitively (34).

AIHA, a diverse condition marked by immune-mediated red blood cell destruction, presents with varying degrees of anemia (35). It is primarily classified based on antibody profiles and temperature reactions. IgG or IgG+C3d antibodies cause warm AIHA (wAIHA), while IgM results in cold agglutinin disease (CAD), causing approximately 20-25% of cases (30). The mixed form combines features of both wAIHA and CAD. The Coombs test, or direct antiglobulin test (36), is the diagnostic gold standard. Our patient's positive results of direct Coombs, anti-C3D, and anti-IgG antibodies confirm the mixed AIHA diagnosis. However, if classified according to the cause, it can be classified into primary and secondary categories (37). Primary AIHA is often associated with immunodeficiency. The distinction between primary and secondary AIHA based on the presence or absence of an underlying disease or condition that exacerbates immune dysregulation and can be further elucidated through genetic sequencing (38). *RAG1* deficiency is a prototypical example of PID (Primary Immunodeficiency Diseases) with a wide range of phenotypic manifestations (38).

AIHA, caused by *RAG1* gene mutations, typically presents as severe, recurrent, and refractory anemia (30). However, the mechanism underlying AIHA may be related to the impairment of receptor editing due to *RAG1* gene mutations. The impairment of the receptor editing caused autoreactive immature B cells to exit the bone marrow, becoming transitional B (TrB) cells that express low levels of BAFF receptors (39), leading to elevated serum BAFF levels. In a lymphopenic environment, this further results in homeostatic proliferation of naive B cells (40). Qing Min and colleagues propose that this homeostatic proliferation leads to the generation of double-negative and memory B cells (41). The double-negative and memory B cells can efficiently differentiate into plasma cells and secrete large amounts of antibodies, including autoantibodies (41). However, it is currently unclear whether this finding is related to the development of AIHA, although elevated autoantibody levels have been observed in mouse models with *RAG* deficiency (26, 40). Furthermore, research has revealed that individuals harboring submorphological *RAG* mutations can generate a diverse array of autoantibodies after exposure to environmental stimuli (26, 30), which may alter the phenotypic expression of autoimmune symptoms. Among the 35 patients reviewed in the literature, except for Coombs-positive cases caused by AIHA, the autoantibodies in 11 patients were unknown, whereas anti-platelet antibodies, anti-neutrophil antibodies, anti-TPO antibodies, anti-IFN-α autoantibodies, and anti-nuclear antibodies were generated in other patients.

According to the World Health Organization, BCG vaccine is absolutely contraindicated in patients with SCID. However, since it is usually administered at birth, most SCID patients in countries where BCG is administered would have been vaccinated before their diagnosis of an immune deficiency (45). It is reported that one in every two SCID patients who receive the BCG vaccine will exhibit manifestations related to the vaccine. These manifestations, including disseminated complications, occur approximately 33,000 times more frequently than that in the general population, while local complications are about 400 times more common (42). In cases of SCID, where prophylactic treatments such as immunoglobulin replacement therapy and antimicrobial agents are commonly used, it is entirely appropriate to commence anti-mycobacterial treatment as early as possible following the diagnosis of SCID. The primary intervention affecting the survival of patients with SCID who had received the BCG vaccine, appears to be immune reconstitution through hematopoietic stem cell transplantation (HSCT). This may be related to the fact that HSCT itself is sufficient as an anti-BCG treatment (42). This implies that there had been neither a previous history of severe infections before age 1 year and 4 months in the patient, nor clinical manifestations of BCG disease or evidence of tuberculosis infection. During the hospital stay, the patient underwent two sputum T-NGS tests, which also did not reveal any signs of tuberculosis infection. Therefore, no clinical treatment was

TABLE 2 Clinical and serological characteristics of patients with distinct phenotypes of RAG deficiency.

CODE	Phenotype	Mutation		Sex	Age at clinical diagnosis	Clinical autoimmunity	Location of granulomas	Other clinical manifestations/ infection	On IvIg	Other immune modulation	HCT	Current status Age (cause of death)	Clinical auto-antibodies (+)	Reference
L/AS-1	Leaky/ atypical SCID	*RAG1*	a.2095(exon2) C>T b.2690 (exon2)G>A	F	1y 4m	AIHA; AN	_	Severe pneumonia; *CMV* infection; *pneumocystis jirovecii* disease	yes	high-dose steroid and plasmapheresis	n.a.	Alive; 1y 7m	Coombs	Present case
L/AS-2	Leaky/ atypical SCID	*RAG1*	a. R669W b. M435V	M	5 y	AIHA; ITP; AN	_	pneumonia; sinus infection; oral candidiasis; vitiligo; psoriasis; Guillain-Barré sy	yes	_	yes; 6y 6m	Alive; 14y 11m	Coombs; anti-platelet ab; anti-neutrophil ab; anti-TPO ab IBD serology+	(11)
L/AS-3	Leaky/ atypical SCID	*RAG1*	a./b. p.R108*	F	3 m	AIHA	_	*CMV*(+); dermatitis	yes; pre-HCT	_	yes; 6.7m	Alive; 2y 7m	Coombs	(11)
L/AS-4	leaky/ atypical SCID	*RAG1*	a./b. K86fs*33	M	11 m	AIHA	_	pneumonia; chronic *CMV* infection; Miller Fisher Syndrome (polyradiculoneuritis with cranial nerve involvement)	yes; pre-HCT	high-dose steroid; rituximab; plasmapheresis	yes;1y 6m	Alive; 3y 4m	Coombs; anti-platelet ab	(11)
L/AS-5	Leaky/ atypical SCID	*RAG1*	a. H65R b. A857V	F	2 y	AIHA	_	adenovirus; *CMV*(+)	yes	_	n.a.	Alive; 3y 1m	Coombs	(11)
L/AS-6	Leaky/ atypical SCID	*RAG1*	a./b. R841W	M	9m	AIHA	_	_	_	_	yes; 10m	Died; 1y 9m	Coombs	(5)
L/AS-7	Leaky/ atypical SCID	*RAG1*	a./b. R474H	F	11y	AIHA; ITP	_	amyloidosis	_	_	n.a.	Alive; 5y	Coombs	(5)
L/AS-8	Leaky/ atypical SCID	*RAG1*	a.1420C (exon2)>T het b.2949(exon2) delA het	F	3y 6m	AIHA	_	recurrent viral and bacterial infections; and nephrotic syndrome	yes; pre-HCT	busulfan; fludarabine and anti-thymocyte globulin	yes; 8 y	Alive	Coombs; Lupus anticoagulant + ANCA; +anti-MPO; Thyroglobulin and thyroid peroxidase Ab; Anti-IFNα Ab	(12)
L/AS-9	Leaky/ atypical SCID	*RAG1*	a.1420C (exon2)>Thet b.2949(exon2) delA het	F	2y	AIHA; AN	_	eczematous rash	yes; pre-HCT	busulfan; fludarabine and anti-thymocyte globulin	HSCT; 2y 5m	Alive	Coombs (IgG and complement); Anti-neutrophil	(12)

(Continued)

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TABLE 2 Continued

CODE	Phenotype	Mutation		Sex	Age at clinical diagnosis	Clinical autoimmunity	Location of granulomas	Other clinical manifestations/ infection	On Ivlg	Other immune modulation	HCT	Current status Age (cause of death)	Clinical auto-antibodies (+)	Reference
													Ab; Anti-IFNα Ab	
L/AS-10	Leaky/ atypical SCID	*RAG1*	a. W522C b. M435V/ M1006 V	M	2y	AIHA; ITP	skin	_	yes; pre-HCT	rituximab	yes;3y	Alive	Coombs; platelet	(5)
L/AS-11	Leaky/ atypical SCID	*RAG1*	a. R396C b. M435V	M	1y 6m	AIHA	_	vasculitis	yes; pre-HCT	steroids; IVIG; cyclophosphamide; alemtuzumab; and/ or rituximab	yes;1y 9m	Died; 2y 8m	Coombs; IFN-α	(5)
L/AS-12	Leaky/ atypical SCID	*RAG1*	a. R561H b. R778Q	M	17y	AIHA	_	_	_	_	_	Alive; 17y	Coombs	(5)
L/AS-13	Leaky/ atypical SCID	*RAG1*	a. N855S b. K992E	M	2y 9m	AIHA	_	hepatitis	_	_	yes; 3y	Alive; 3y	Coombs	(5)
L/AS-14	Leaky/ atypical SCID	*RAG1*	a. R142* b. T477S	F	2y 10m	AIHA; ITP; AN	_	_	_	_	yes; 3y 3m	Died; 3y 4m	Coombs; platelet	(5)
L/AS-15	Leaky/ atypical SCID	*RAG1*	a. R15L b. H735Q	F	n.a.	AIHA; ITP	_	_	_	_	yes; 1y 4m	Alive; 3y 9m	Coombs; thyroid (TPO)	(5)
L/AS-16	Leaky/ atypical SCID	*RAG1*	n.a.	M	14y	AN; ITP; AIHA	_	vitiligo; IBD	_	_	yes; 14y	Died; 14y	Coombs	(5)
CID-1	CID-G/AI	*RAG1*	a. R474C b. K983Nfs*9	F	2y	AIHA; AN	_	otitis media; RSV bronchiolitis; severe varicella with periorbital cellulits/keratitis	yes; pre-HCT	_	yes; 2 and 4 y	Alive; 3m	Coombs;anti-neutrophil antibody+	(11, 12, 13, 22)
CID-2	CID-G/AI	*RAG1*	a./b.S117fs	M	8y	AIHA	skin	pneumonias; liver abscess; Mycobacterial osteomyelitis; herpes-zoster; diarrhea from rotavirus viral encephalitis after yellow fever vaccine; varicella	yes; pre-HCT	_	yes;8 y	Alive; 8y	Coombs	(10)
CID-3	CID-G/AI	*RAG1*	a. K86fs*33 b. H65R	F	3y	ITP; AIHA	lung	recurrent sinopulmonary infections	yes; pre-HCT	corticosteroids and infliximab	yes; 18y	Alive; 20y	Coombs; thyroid (TPO)	(13)

(Continued)

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TABLE 2 Continued

CODE	Phenotype	Mutation		Sex	Age at clinical diagnosis	Clinical autoimmunity	Location of granulomas	Other clinical manifestations/ infection	On IvIg	Other immune modulation	HCT	Current status Age (cause of death)	Clinical auto-antibodies (+)	Reference
								(*Penicillium*; *Corynebacterium propinquum*; and *Pseudomonas aeruginosa*); viral infections (shingles); and vitiligo)						
CID-4	CID-G/AI	*RAG1*	a./b. S480G	M	6y	AIHA; AN	_	Enteropathy; Glomerulonephritis; atherosclerosis; generalized urticaria	yes; pre-HCT	rituximab	yes; 9y 6m	Died; 10y	Coombs; neutrophil	(14)
CID-5	CID-G/AI	*RAG1*	a./b.[His65Arg]	M	7y	AIHA; ITP	skin	Severe varicella infection; generalized scabies; recurrent bronchitis	yes; pre-HCT	rituximab	yes; 7y 6m	Alive	Coombs	(14)
CID-6	CID-G/AI	*RAG1*	a./b.2095C>T	M	6y	AIHA	skin, lung, liver, bone, pancreas, testes	otitis media; pallor and splenomegaly	yes; pre-HCT	high-dose steroid; rituximab; plasmapheresis	n.a.	Died	Coombs; ANA; dsDNA	(15)
CID-7	CID-G/AI	*RAG1*	a. R474C b. R975W	F	9y	AIHA; ITP	skin	_	yes; pre-HCT	rituximab	yes; 20y	Died; 21y	Coombs; platelet	(5)
CID-8	CID-G/AI	*RAG1*	a. W522C b. H994R	M	3y	AIHA	_	vasculitis	yes; pre-HCT	steroids; cyclophosphamide; alemtuzumab; and/ or rituximab	yes;5y	Died; 6y	Coombs	(5)
CID-9	CID-G/AI	*RAG1*	a./b. R764C	F	16y	AIHA; ITP	skin, bone	splenomegaly; hypergamma; globulinemia; pancytopenia	_	_	n.a.	Alive	Coombs	(16)
CID-10	CID-G/AI	*RAG1*	a. R841Q b. F974L	F	6m	AIHA; ITP; AN	_	vasculitis; myopathy; central demyelinating neuropathy	yes	glucocorticoids; intravenous immunoglobulin; enoxaparin; and rituximab	died	Died; 2y	Coombs; platelet	(17)
CID-11	CID-G/AI	*RAG1*	a./b. H65R	F	15y 7m	AIHA; AN	_	alopecia areata; thyroiditis	yes; pre-HCT	_	yes; 17y	Alive; 18y	Coombs; IFN-α; thyroid (TPO/TG)	(5)

(Continued)

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TABLE 2 Continued

CODE	Phenotype	Mutation		Sex	Age at clinical diagnosis	Clinical autoimmunity	Location of granulomas	Other clinical manifestations/ infection	On Ivlg	Other immune modulation	HCT	Current status Age (cause of death)	Clinical auto-antibodies (+)	Reference
CID-5	CID-G/AI	*RAG1*	a./b. R507G	M	5y	AIHA; AN	_	hepato-splenomegaly	yes; pre-HCT	high-dose steroid; rituximab; plasmapheresis	yes; 5y 3m	Alive; 8y	Coombs; neutrophil	(18)
CID-13	CID-G/AI	*RAG1*	a./ b. K86VfsX33	M	4y	AIHA; ITP; AN	_	_	yes; pre-HCT	_	yes; 4y	Alive; 7y 8m	Coombs	(5)
CID-14	CID-G/AI	*RAG1*	a./b. N855S	M	11y	AIHA	_	enteropathy	none	_	n.a.	Died; 11y	Coombs; enterocyte/ goblet cell	(5)
CID-15	CID-G/AI	*RAG1*	a./b. G816R	M	7y 6m	AIHA	_	sclerosing cholangitis	_	_	n.a.	Alive; 9y 6m	Coombs	(5)
CID-16	CID-G/AI	*RAG1*	a.1187G>A (Arg396His) b.1566G>T (Trp522Cys)	F	5y	AIHA	skin	herpes simplex virus (HSV) gingivostomatitis; bacterial pneumonia; and cutaneous abscess	yes; pre-HCT	high-dose steroid; rituximab; plasmapheresis	yes	Alive	Coombs	(19)
CID-17	CID-G/AI	*RAG1*	a.M435V (1303AG) b.R699W (2095C¡T)	M	10m	AIHA; ITP; AN	_	vitiligo; psoriasis; tetanus; diphtheria and Guillain-Barré syndrome	yes; pre-HCT	high-dose steroids; infliximab; and cyclophosph amide	yes; 6y	Alive	Coombs	(20)
CID-18	CID-G/AI	*RAG1*	a.M435V (1303AG) b.R699W (2095C¡T)	M	8y	AIHA; AN	skin	varicella infection; pneumonia; tuberculosis infections; parasitic infections	yes; pre-HCT	high-dose steroids; infliximab; and cyclophosph amide	yes; 8y	Died; 8y	Coombs; pANCA; ANA	(23)
SCID-1	SCID	*RAG1*	a. N766l b.K86VfsX33	M	13y	AIHA; AN	_	thyroiditis; hepatitis; urticaria	_	_	yes; 5m	Alive; 19y	Coombs	(5)
SCID-2	SCID	*RAG1*	a./ b. K86VfsX33	F	1m	AIHA	_	_	yes; pre-HCT	_	yes;1y 3m	Alive 10y5m	Coombs	(5)
SCID-3	SCID	*RAG1*	del AA368–369	M	11m	AIHA; thrombocytopenia	_	hepatosplenomegaly; *CMV* infection	yes; pre-HCT	ganciclovir; foscarnet; and *CMV* hyperImmunoglobulin	yes;1y 4m	Alive	Coombs	(21)

(Continued)

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TABLE 2 Continued

CODE	Phenotype	Mutation		Sex	Age at clinical diagnosis	Clinical autoimmunity	Location of granulomas	Other clinical manifestations/ infection	On Ivlg	Other immune modulation	HCT	Current status Age (cause of death)	Clinical auto-antibodies (+)	Reference
SCID-4	SCID	*RAG1*	g.8366-8367insT, c.3198-3199insT, p.L1025FfsX39 g.6472C>T, c.1304C>T, p.R394 W	F	45d	AIHA;	_	hepatosplenomegaly; erythroderma; lymphadenopathy	_	_	_	_	Coombs	(22)
OS-1	OS	*RAG1*	g.8366-8367insT, c.3198-3199insT, p.L1025FfsX39	F	82d	AIHA; ITP;	_	_	_	_	_	_	Coombs	(22)
OS-2	Omenn syndrome	*RAG1*	g.8366-8367insT, c.3198-3199insT, p.L1025FfsX39 g.7481G>A, c.2313G>A, p.C730Y	M	60d	AIHA;	_	hepatosplenomegaly; erythroderma; lymphadenopathy	_	_	_	_	Coombs	(22)
OS-3	Omenn syndrome	*RAG1*	g.7862C>T, c.2694C>T, p.A857 V	F	120d	AIHA;	_	hepatosplenomegaly; erythroderma; lymphadenopathy	_	_	_	_	Coombs	(22)

AIHA (autoimmune hemolytic anemia), ITP (immune thrombocytopenia), AN (autoimmune neutropenia), *CMV* (cytomegalovirus), *RSV* (respiratory syncytial virus), TPO (thyroperoxidase antibody), HCT (Hematopoietic cell transplantation).

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administered for BCG disease. However, the patient received IVIG as immunosupportive therapy, and antibiotic treatment.

In patients with *RAG* deficiency, HSCT, followed by genetic reconstitution, is the only definitive therapeutic option (27, 43). However, suitable donors for this purpose are not always available. According to our literature-based statistics, 28 patients underwent HSCT, with eight fatalities occurring post-transplantation and three before HSCT. Nonetheless, the literature suggests that only 50% of patients undergoing HSCT survive this procedure (44). Additionally, HSCT for CID administered before 3.7 months of age has been shown to yield more favorable outcomes (28, 38). However, the median age in our cohort was 3.75 years (range, 1 months – 20 years). This delay may stem from the need to arrange for patient transfers to other hospitals for treatment and the heightened risk of infections and tissue damage. It is well-established that infections and tissue damage can negatively affect HSCT outcomes (43). Thus, the management of patients with subformal RAG deficiencies is not as straightforward as that of patients with typical SCID. Autoimmune complications frequently necessitate immunosuppressive and immunomodulatory medications, further increasing the risk of infection. Accordingly, we observed a lack of response to first-(primarily IVIG and steroids) or second-line therapies (primarily rituximab) in most patients. Our patient was also treated with these drugs along with hormone and plasma exchange therapy. Although the hemolytic symptoms of the patient improved after treatment, serious opportunistic infections, giant cells, and *Pneumocystis jirovecii* were present during this period. The patient is now in good condition and is still undergoing relevant immunization counseling. Rituximab is a highly effective and safe alternative treatment for ITP and AIHA in patients with common variable immunodeficiency (41).When patients are diagnosed at a later age, there is an increased risk of developing graft-versus-host diseases. Furthermore, partial development of T and B cells requires a myeloablative preconditioning regimen to prevent graft rejection (46).

In conclusion, we report a case of *RAG1* mutation reported in China, which initially manifested as AIHA. We also present literature review that outlines the diverse clinical phenotypes observed in *RAG1* mutations presenting with AIHA. For such children, hormones and IVIG can be used as emergency treatments for AIHA; however, these drug control measures increase the risk of opportunistic infections, making HSCT the ultimate necessary choice.

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 authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shengjing Hospital of China Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/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

XC: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. CJ: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. JY: Data curation, Methodology, Writing – review & editing. WS: Data curation, Formal analysis, Methodology, Writing – review & editing. TS: Data curation, Methodology, Writing – review & editing.WX: Data curation, Methodology, Supervision, Visualization, Writing – review & editing. KY: Funding acquisition, Resources, Software, Validation, Visualization, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Natural Science Foundation of China Youth Fund (grant number 81501292); China Postdoctoral Fund Surface Project (grant number 2017M611285); and Basic Research Program of Liaoning Province (grant number 2022JH2/101500053). The sponsors were not involved.

Acknowledgments

The authors would like to thank the research group at the Department of Pediatrics of Shengjing Hospital, affiliated with China Medical University. The authors also thank Editage (www.editage.cn) for the English language editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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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.2024.1498066/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Evidence of AIHA **(A)** Bone marrow cytology: Significant proliferation and activation of bone marrow, with decreased granulopoiesis, robust erythropoiesis dominated by mid-to-late stage erythroblasts, frequently exhibiting abnormal nuclei. These abnormalities include poikilocytosis (irregularly shaped red blood cell precursors), flower-like erythroblasts, polychromasia, and direct and indirect mitotic figures. **(B)** Value of hemoglobin levels, reticulocyte count, and reticulocyte percentage. Dotted lines indicate the reference range.

SUPPLEMENTARY FIGURE 2
Specific trends and values of inflammatory markers **(A)** C-reactive protein, interleukin-6 and procalcitonin. **(B)** White blood cell count and serum ferritin. Dotted lines indicate the reference range.

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