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Deciphering the landscape of immunohematology: Enhancing our understanding and management of hematological disorders through advances in immunology and genetics

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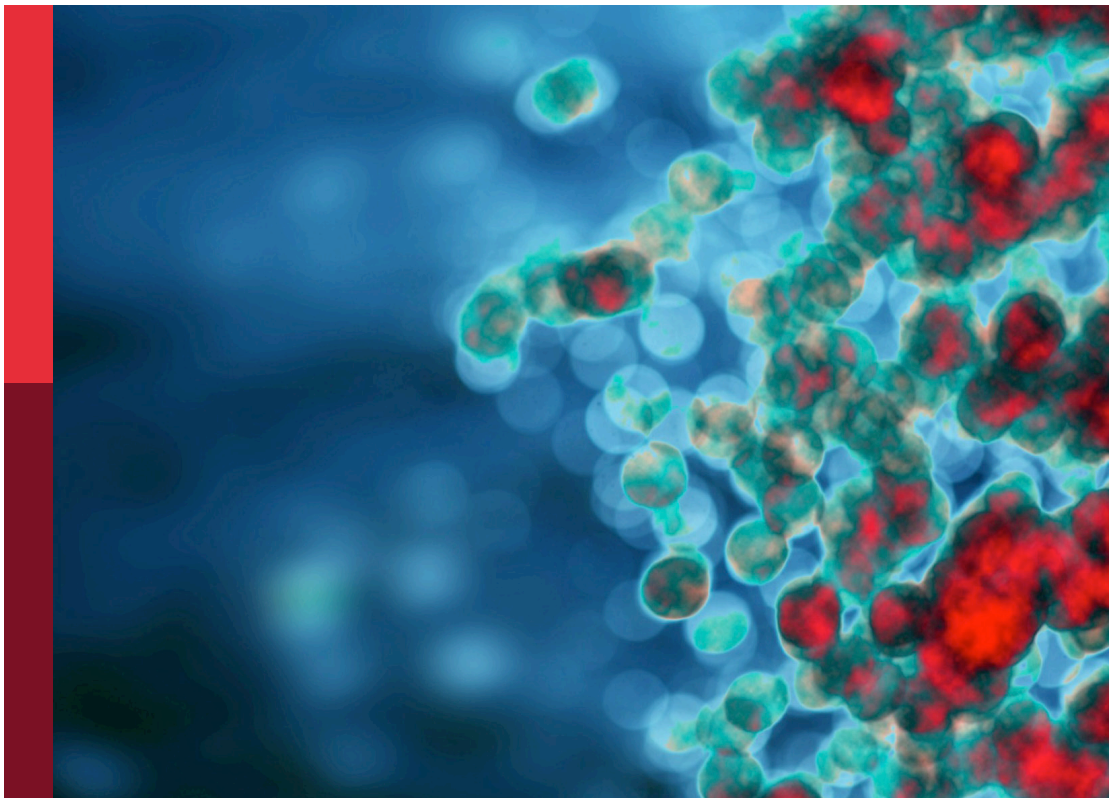

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Deciphering the landscape of immunohematology: Enhancing our understanding and management of hematological disorders through advances in immunology and genetics

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CASE REPORT
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Case Report: Deficiency of Adenosine Deaminase 2 Presenting With Overlapping Features of Autoimmune Lymphoproliferative Syndrome and Bone Marrow Failure

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Deficiency of adenosine deaminase 2 (DADA2) is an autosomal recessive disease associated with a highly variable clinical presentation, such as vasculitis, inflammation, and hematologic manifestations. Some associations of clinical features can mimic autoimmune lymphoproliferative syndrome (ALPS). We report a case of a female patient who fulfilled the 2009 National Institute of Health revised criteria for ALPS and received a delayed diagnosis of DADA2. During her childhood, she suffered from autoimmune hemolytic anemia, immune thrombocytopenia, and chronic lymphoproliferation, which partially responded to multiple lines of treatments and were followed, at 25 years of age, by pulmonary embolism, septic shock, and bone marrow failure with myelodysplastic evolution. The patient died from the progression of pulmonary disease and multiorgan failure. Two previously unreported variants of gene ADA2/CECR1 were found through next-generation sequencing analysis, and a pathogenic role was demonstrated through a functional study. A single somatic STAT3 mutation was also found. Clinical phenotypes encompassing immune dysregulation and marrow failure should be evaluated at the early stage of diagnostic work-up with an extended molecular evaluation. A correct genetic diagnosis may lead to a precision medicine approach consisting of the use of targeted treatments or early hematopoietic stem cell transplantation.

Keywords: bone marrow failure (BMF), primary immune regulatory disorders (PIRDS), autoimmune lymphoproliferative syndrome (ALPS), next-generation sequencing (NGS), DADA2, inborn errors of immunity (IEI)

INTRODUCTION

Deficiency of adenosine deaminase type 2 (DADA2) is an autosomal recessive disease caused by loss-of-function mutations of the ADA2/CECR1 gene, which encodes adenosine deaminase type 2 (ADA2) (1). ADA2 is partially homologous to adenosine deaminase type 1 (ADA1) (1), which is involved in a key step of purine metabolism by breaking down adenosine (Ado) and 2′-deoxyadenosine (dAdo) to deoxyinosine (2, 3). However, ADA2 has a distinct 59-kDa structure and a lower affinity to Ado and dAdo, accounting for a limited role in purine metabolism and additional non-redundant functions. In fact, one type of adenosine deaminase cannot compensate for the absence of the other enzyme, as ADA1 deficiency results in severe combined immunodeficiency (1). Unlike ADA1, ADA2 forms homodimers with a molecular weight of ~110 kDa (3), and it is produced by activated monocytes, macrophages, and dendritic cells during inflammatory response, as in patients with an autoimmune disease or infections (1, 4–7). For proper translocation to extracellular space, ADA2 needs to be N-glycosylated (8). Upon release, ADA2 binds to the surface of various immune cells, possibly through the PRB domain (9), to induce the T-cell-dependent differentiation of monocytes into macrophages and a growth factor activity, which is partially unknown. ADA2 deficiency is associated with monocyte polarization to M1 macrophages, which are known to induce inflammation and tissue damage and increase the release of proinflammatory cytokines (1, 9, 10).

The clinical onset of DADA2 was reported before 1 and 10 years of age in 24 and 77% of patients, respectively, with a mortality rate of 8% before the age of 30 years. The clinical features of 161 patients have been retrospectively reported by Meyts et al. in 2018 (1), showing a highly variable and misleading clinical presentation due to vasculitis/vasculopathy of small- and medium-sized arteries. Skin manifestations were reported in >75% of patients, while neurological involvement with ischemic or hemorrhagic stroke was present in 50%, with potential underestimation when presenting as transient ischemic attacks (1). Consistent with a systemic inflammatory process, most patients experience recurrent fever, myalgia, arthralgia, serositis, and elevated inflammatory markers such as erythrocyte sedimentation rate and C-reactive protein (3). Less commonly, gastrointestinal and renal involvement, arthritis, and myositis were reported (11, 12). In addition to the mentioned inflammatory features, significant hematologic and immunologic involvement has been described recently. Hypogammaglobulinemia and a common variable immune deficiency (CVID) phenotype have been described in 25% of patients, with or without concurrent findings of vasculopathy (1, 3). Clonal lymphoproliferation (13), generalized lymphoadenopathy (>10%), and splenomegaly (up to 30%) were also reported. Other later reports described further hematological manifestations, including pure red cell aplasia (PRCA), and cytopenia affecting one or more cell lineages (12, 14). The specific association of symptoms might resemble autoimmune lymphoproliferative syndrome (ALPS), as described in a report by Alsultan (15). The severity of the marrow failure of the patient may lead to the indication of hemopoietic stem cell transplantation (HSCT), which represents the only curative option for congenital diseases (16). HSCT has been used in patients with a severe phenotype (10, 17–19) that did not respond to medical treatments such as tumor necrosis factor (TNF) inhibitors, which represent the best option in controlling fever episodes and vasculopathy and in preventing stroke (1, 20, 21).

We describe a case of a young woman with a long history of ALPS during childhood followed by rapid evolution to bone marrow failure, which resulted from carrying a novel pathogenic genotype of the ADA2/CECR1 gene.

CASE PRESENTATION

The clinical history of the patient is summarized in **Figure 1**. Apart from chickenpox and measles that occurred despite specific vaccination, no significant clinical issues were reported during early childhood. Another center followed up with her since the age of 5 years after an episode of trilinear cytopenia associated with splenomegaly. The marrow examination demonstrated good cellularity. No detailed information on therapeutic approaches was available at that time. Her family reported that she was treated with high-dose steroid therapy, transfusions, anti-thymocyte globulin, and cyclosporine A, with a complete recovery on platelet count and a partial response on other cell lines.

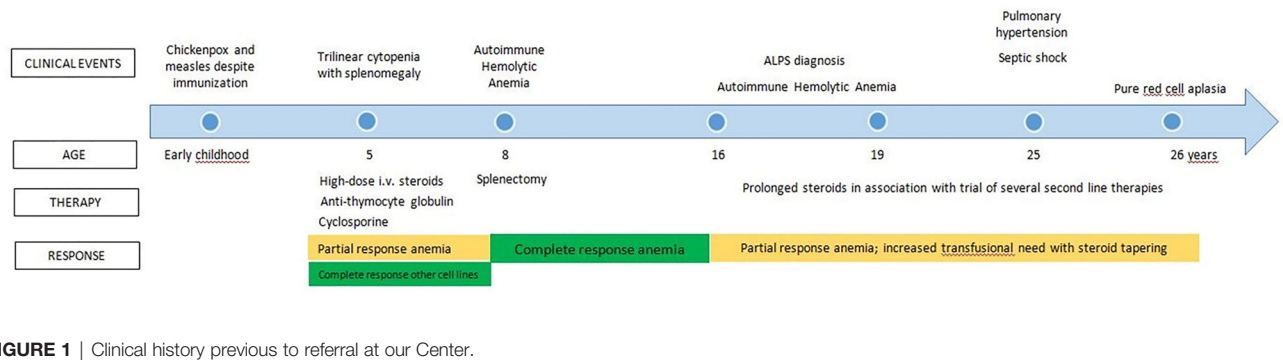

FIGURE 1 | Clinical history previous to referral at our Center.

Three years later, an episode of acute autoimmune hemolytic anemia (AIHA) was successfully treated with splenectomy. At the age of 16, she developed several new episodes of AIHA associated with chronic lymphoproliferation and high values of T cell receptor $\alpha\beta^+$ $CD4^-$ $CD8^-$ double-negative T cells (DNT). Defective Fas-mediated T-cell apoptosis was demonstrated in two different laboratories in order to obtain diagnostic confirmation. She received a diagnosis of ALPS, according to the 2009 National Institute of Health (NIH) revised criteria (22). Along with steroid treatment, she received other lines of therapy, such as cyclophosphamide, rituximab, micophenolate mofetil, azathioprine, vincristine, and, lastly, tacrolimus. All these therapeutic options, performed over about 10 years, only resulted in a partial response of steroid-dependent AIHA. In fact, attempts to withdraw steroids were followed by an increased transfusion need. At that stage, the bone marrow examination was still normal.

At the age of 25, during follow-up at the other center, she received a chest X-ray, which revealed a potential lung nodule that required further evaluations. A computed tomography scan and lung scintigraphy showed features of pulmonary embolism, leading to a diagnosis of pulmonary hypertension without any previous symptom or thrombotic event. Thrombophilia screening demonstrated protein S deficiency; therefore, apixaban prophylaxis was started. Meanwhile, she developed septic shock from *Streptococcus gallolyticus*, requiring intensive care. One year later, she was referred to our center for a second opinion because of worsening anemia despite the steroid and tacrolimus treatment.

Table 1 shows the significant results of a blood examination performed on admission to our center. Hyporegenerative anemia and mild neutropenia were found. An immunological re-evaluation confirmed that her case fulfilled the 2009 NIH ALPS criteria, but with a significant reduction in immunoglobulin levels, and her plasma-soluble FAS ligand levels were normal. The trephyne biopsy showed severe erythroid hypoplasia, associated with normal myeloid/lymphoid cellularity and megakaryocytes. The marrow progenitor assay demonstrated reduced numbers of burst forming unit-erythroid and colony-forming unit for granulocytes and macrophages. The addition of the plasma of the patient to heterologous marrow cell precursors inhibited cellular growth and differentiation, possibly suggesting a humoral inhibitory effect on the marrow progenitor cells. Based on the clinical and laboratory findings and on the unsatisfactory control of the clinical symptoms, tacrolimus was substituted by sirolimus, while the steroids were slowly tapered off. Due to the absence of data on immunoglobulin levels before rituximab administration, it was not possible to determine whether hypogammaglobulinemia was either treatment- or disease-related, although the previously failed attempt to immunize against measles and chickenpox raised the suspicion of a previous CVID phenotype. Therefore, a program of regular subcutaneous immunoglobulin administration was started in order to reduce any risk of secondary infections related to the immunosuppressive treatment. Iron chelation treatment was also started due to elevated ferritin levels secondary to previous intensive transfusion support. Since sirolimus did not produce any response, erythropoietin was additionally administered weekly. At that stage, the patient was continuously offered HSCT, but it was strongly refused.

At 5 months after being referred to our center, the patient developed severe neutropenia and fever, requiring hospitalization. The trephine biopsy demonstrated severely reduced

TABLE 1 | Significant laboratory tests at admission in our center.

	Results	Reference range
Hemoglobin	9.2 g/dl	11.5–16.5
Lactic dehydrogenase	983 U/L	84–480
Haptoglobin	<2 mg/dl	15–160
Total lymphocyte count	7,150/mmc	3,600–9,800
Lymphocyte subsets		
$CD3^+TCR\alpha\beta^+CD4^-CD8^-$ DNT cells	5.1% (365/mmc)	<1.5% of total lymphocytes
B cells $CD\ 19^+$	2.6% (186/mmc)	6–19%
B cells $CD27^+$ ($CD19^+$ tot)	15.9% (1,137/mmc)	>15%
$CD3CD25^+/CD3HLADR^+$ ratio	0%	>1
$CD3^+TCR\ \alpha\beta^+\ CD4^-CD8^-\ B220^+$ cells[a]	82.7% (5,913/mmc)	<60%
Autoimmune lymphoproliferative syndrome (ALPS) biomarkers		
Plasma sFASL levels	0.5 pg/ml	0 >200 in ALPS
Elevated plasma interleukin-10 levels	<1 pg/ml	<1 pg/ml >20 in ALPS
Elevated serum or plasma vitamin B12 levels	374 ng/L	191–663 >1,500 in ALPS
Elevated plasma interleukin-18 levels	5,750 pg/ml	36–258 >500 in ALPS
Immunoglobulin G	254 mg/dl	700–1600
Immunoglobulin A	13 mg/dl	70–400
Immunoglobulin M	299 mg/dl	40–230

[a]*Increased B220+ T lymphocytes are significantly associated with ALPS with good specificity (23–25).*

granulocytopoiesis and erythropoiesis and dysmegakaryocytopoiesis. The patient quickly developed an overwhelming hyperinflammatory syndrome and, due to the progressive worsening of her respiratory function, she was admitted to the intensive care unit. Unfortunately, despite extracorporeal membrane oxygenation, the patient died from progressive multiorgan failure and right ventricular cardiac thrombosis.

DIAGNOSTIC ASSESSMENT

During the follow-up at our center, a next-generation sequencing (NGS) panel that included genes related to both congenital marrow failure and immune dysregulation syndromes (26, 27) was applied to our proband (II-1 in **Figure 2**). Unfortunately, the results were released only a few days before the death of the patient and showed two germline mutations of the ADA2/CECR1 gene (OMIM#607575; transcript NM_001282225.2): (i) c.563T>C, leading to p.Leu188Pro, reported also by Michniacki in 2018 in association with DADA2 (28), and (ii) c.559A>C, leading to p.Thr187Pro, previously unreported. Based on the American College of Medical Genetics and Genomics criteria (29), both variants are classified as having a "likely pathogenic" effect and, consistent with the autosomal recessive inheritance of DADA2 (OMIM#615688), they turned out to be inherited by her father and mother, respectively (**Figure 2**). These observations confirmed the causal role of the ADA2/CECR1 genotype of our patient on her condition. Both mutations were also found in the sister of the patient (II-2 in **Figure 2**), who displayed a clinical history of polyarticular arthritis of the small joints of the hands, along with Raynaud's phenomenon, hip and knee arthralgia, mild leukopenia, and mild thrombocytopenia. After the result of the genetic test, a targeted immunological screening revealed hypogammaglobulinemia and increased values of DNT cells (2.5%) in the mother of the patient (I-2 in **Figure 2**).

Since none of the ADA2/CECR1 variants found in our patient were reported in any database of pathogenic mutations at the moment of her genetic diagnosis, a functional analysis on peripheral monocytes was performed to test their effect on ADA2 activity. These cells were isolated by adherence, after peripheral blood mononuclear cell Ficoll–Paque separation, and were then cultured in phosphate-buffered saline with exogenous adenosine (Sigma Aldrich) with or without ADA1 inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (Sigma Aldrich) for 4 h at 37°C with 5% of CO_2. The supernatants were collected, and the activity enzyme was indirectly evaluated in high-performance liquid chromatography through the measurement of the adenosine-derived products (inosine and hypoxanthine) as a surrogate marker of enzyme activity (20). As shown in **Figure 3**, no adenosine metabolites were detectable in our patient (Pt 1), thus suggesting a complete loss of enzymatic activity. Consistently, both the patient and her sister presented compound heterozygosity for the same variants, and we could demonstrate a complete absence of inosine, the most important adenosine-derived product.

Finally, a heterozygous pathogenic somatic mutation on STAT3 (p.Lys658Arg) was also identified in our patient. The same mutation was found neither in her parents nor in her sister, and its somatic origin was confirmed by its absence in the skin fibroblasts of the patient.

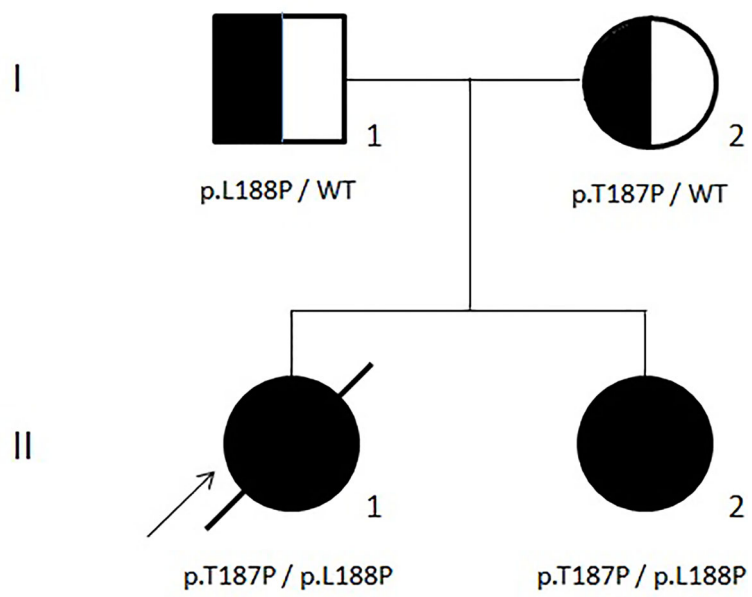

FIGURE 2 | Family tree.

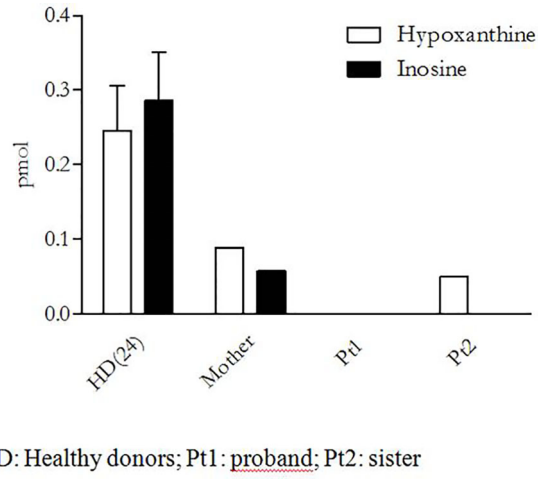

FIGURE 3 | Functional assay of ADA2 activity.

DISCUSSION

The clinical history of the patient was characterized by symptoms and laboratory findings fulfilling the 2009 NIH ALPS diagnostic criteria (22), followed, in early adulthood, by the onset of more specific features of DADA2, such as vasculopathy, marrow failure, and hyperinflammatory symptoms (1).

ADA2/CECR1 missense, frameshift mutations, splicing defects, or deletions have been described as pathogenic and were distributed in all different structural domains (3, 11, 30, 31). In a recent work, Lee et al. performed a literature review and a genotype comparison of vasculitis and hematologic phenotypes in DADA2. In the manuscript, the ADA2/CECR1 mutations were clustered in groups according to their predicted residual enzymatic activity. The prevalence of PRCA or marrow failure features was greater in groups according to their lower predicted enzymatic activity (<3% residual enzymatic activity), in particular, with insertion–deletion mutations (indels), early-termination mutations, and missense mutations, including Leu188Pro, which we found in our patient (32). However, the pathogenic mechanism of the residual enzymatic activity toward vasculitis or marrow failure remains to be determined.

The two novel ADA2/CECR1 mutations found in our case could explain both the ALPS and DADA2 phenotypes.

Unusual phenotypes with features overlapping both rheumatological and hematological disorders have been already reported not only in DADA2 patients but also in other autoinflammatory/autoimmune disorders (23), which can show the expansion of DNT cells and other ALPS markers, making the diagnosis particularly challenging. Similarly, a significant proportion of ALPS patients may also present with a consistent inflammatory phenotype (23).

In the first phases of the disease, the patient fulfilled the NIH 2009 ALPS criteria (22). However, some typical ALPS biomarkers, such as sFAS, IL-10, and vitamin B12, resulted to be normal. The immunoglobulin levels in this patient were not a reliable diagnostic criterion due to a previous rituximab treatment (33). In addition, although anemia was initially secondary to peripheral autoimmune hemolysis with normal marrow cellularity, in the following years, it became hyporegenerative with erythroid hypoplasia and tested negative in both direct and indirect antiglobulin tests, a feature atypical of ALPS. Over the past 10–15 years, improvements in genomic technologies have led to the description of a number of monogenic disorders mimicking ALPS. These rare conditions, defined as CVID or ALPS-like phenotypes, clinically resemble ALPS and, therefore, are often misdiagnosed, highlighting the urgent need to revise the NIH ALPS diagnostic criteria based on increased knowledge of the pathogenic mechanisms and biomarkers of such disorders (23–25, 34, 35). Therefore, an earlier genetic diagnosis should be performed in all patients with immune dysregulation to define a more precise therapeutic strategy and to make a proper assessment in case of stem cell transplantation. The most important signal for correctly diagnosing and treating this patient was the progressive evolution of the clinical phenotype over time, with prevalent inflammatory features, vasculitis, and bone marrow failure with PRCA, although such signs and symptoms of DADA2 and the disease itself were still mostly unknown at that time.

In our patient, marrow involvement, initially characterized by PRCA, evolved into severe trilinear marrow failure, in keeping with the concept that DADA2 phenotypes likely represent a continuum rather than different categories (32).

A colony-forming unit assay clearly showed not only the reduced growth of marrow progenitor cells but also an inhibitory effect on the plasma of the patient with heterologous marrow progenitors, suggesting a potential contribution of humoral immunity possibly related to immune dysregulation. Indeed the pathogenesis of marrow failure in ADA2 deficiency remains largely not understood. An ADA2 knocked-down zebrafish model displays neutropenia, thus supporting an intrinsic role of ADA2 in normal hematopoiesis (12). On the other hand, human ADA2 was shown to have an *in vitro* growth factor activity (7) whose absence may have contributed to the development of marrow failure.

In addition, the coexistence of strongly diminished ADA2 activity with an oligosymptomatic phenotype in the sister can be explained by well-known intrafamilial phenotypic variability despite the same underlying homozygous mutations (11,19,30,36–40). However, even if individuals with biallelic ADA2/CECR1 pathogenic variants were reported to have remained asymptomatic until adulthood or to have never developed clinical manifestations of DADA2 (41), the sister of our patient is currently following up with another adult rheumatology center.

A gain-of-function, likely pathogenic somatic heterozygous STAT3 somatic mutation, was also shown by the NGS panel in the marrow cell of our patient. This variant had not been previously reported. The STAT3 gene (42) encodes a transcription factor activated in response to cytokine signaling, and germline gain-of-function STAT3 mutations were reported after whole-exome sequencing and whole-genome sequencing

studies as new potential genetic drivers of ALPS-like phenotypes (43, 44). On the other hand, somatic heterozygous STAT3 gain-of-function mutations are also reported in literature in association with myelodysplastic syndrome (45–47). We found this mutation only in cells derived from the hematopoietic lineage, while skin fibroblasts resulted as wild type for STAT3. Unfortunately, it is not possible to define the contribution of the STAT3 mutation in our patient due to the unavailability of marrow samples and genetic tests at the onset of her symptoms. We can only speculate that such mosaicism might have been either a sign of a myelodysplastic evolution or present since diagnosis, contributing to the onset of the ALPS phenotype, similar to somatic mutations in the FAS gene (48, 49).

The overlap between marrow failure and immune dysregulation has recently been documented by our group in a large study cohort of patients (27). This reinforces the idea that young patients with marrow failure should undergo early immunological screening and be offered genetic tests by either extended next-generation sequencing panels (50), which include genes leading to primary immune deficiencies, or unbiased whole-exome sequencing, when available. In fact, improvements in diagnostic accuracy may lead to an early targeted therapy. In our patient, an earlier diagnosis of DADA2 could have led to a more prompt and tailored treatment with anti-TNF alpha, potentially improving the inflammatory phenotype and controlling the progression of the disease (1, 21, 32). The previous indication to splenectomy could have been further evaluated, balancing rewards and risks as infectious risk, if a genetic diagnosis was available at that moment. She experienced an episode of sepsis and a hyperinflammation evolving in fatal multiorgan failure with cardiac thrombosis: the association of splenectomy and several immunosuppressive treatments could have represented the risk factors for such complications. HSCT, even in the absence of a genetic diagnosis, could have prevented the fatal progression of other co-morbidities, but the patient strongly refused it. This procedure may be considered earlier for patients with severe hematologic presentation (10, 17–19, 32).

In conclusion, this case report suggests that clinical phenotypes encompassing immune dysregulation and marrow failure should be evaluated at the early stage of diagnostic work-up with an extended molecular evaluation that includes genes that cause both groups of disorders. Proper genetic diagnosis may lead to precision medicine approach and targeted treatments.

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: (https://www.ncbi.nlm.nih.gov/clinvar/) VCV000973671.1, VCV000973614.1, and VCV000421491.2.

AUTHOR CONTRIBUTIONS

GD and MM conceived the presented idea. GD, RC, EP, ER, SM, FiP, MG, CD, and MM reviewed the clinical information presented. MG, CD, and MM oversaw the writing, data collection, and editing process. IC, MG, FF, CD, and MM provided critical review of the manuscript. PT and ML performed immunological assays. AG and IC performed genetic diagnosis. FeP, RC, and FS performed functional assay on a research basis. All authors contributed to the article and approved the submitted version.

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Digenic Inheritance: Evidence and Gaps in Hemophagocytic Lymphohistiocytosis

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Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory disorder characterized by the inability to properly terminate an immune response. Familial HLH (FHLH) and related immune dysregulation syndromes are associated with mutations in the genes *PRF1, UNC13D, STX11, STXBP2, LYST, AP3B1*, and *RAB27A*, all of which are required for the assembly, exocytosis, and function of cytotoxic granules within CD8+ T cells and natural killer (NK) cells. Loss-of-function mutations in these genes render the cytotoxicity pathway ineffective, thereby failing to eradicate immune stimuli, such as infectious pathogens or malignant cells. The resulting persistent immune system stimulation drives hypercytokinemia, ultimately leading to severe tissue inflammation and end-organ damage. Traditionally, a diagnosis of FHLH requires the identification of biallelic loss-of-function mutations in one of these degranulation pathway genes. However, this narrow definition fails to encompass patients with other genetic mechanisms underlying degranulation pathway dysfunction. In particular, mounting clinical evidence supports a potential digenic mode of inheritance of FHLH in which single loss-of-function mutations in two different degranulation pathway genes cooperate to impair pathway activity. Here, we review the functions of the FHLH-associated genes within the degranulation pathway and summarize clinical evidence supporting a model in which cumulative defects along this mechanistic pathway may underlie HLH.

Keywords: hemophagocytic lymphohistiocytosis, digenic, degranulation, variants, cytotoxic lymphocyte, natural killer cell

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INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a hyperinflammatory syndrome mediated by an ineffective yet hyperactive immune response. Patients with HLH appropriately initiate an immune response in the presence of an immunogenic stimulus, but they display impaired eradication of these stimuli, resulting in persistent immune cell activation and excessive cytokine secretion. This in turn can lead to fatal end-organ damage in the absence of treatment aimed at controlling hyperinflammation (1). Based on diagnostic criteria proposed by the Histiocyte Society in conjunction with the HLH-2004 clinical trial, HLH should be considered in patients presenting with at least five of eight clinical features including fever, splenomegaly, multi-lineage cytopenias,

hypofibrinogenemia or hypertriglyceridemia, hyperferritinemia, hemophagocytosis, elevated soluble CD25, and low or absent natural killer (NK) cell activity (2).

Historically, HLH has been subdivided into primary, or familial HLH (FHLH), and secondary, nonfamilial HLH (3, 4). FHLH most commonly presents during infancy or early childhood, with approximately 70% of patients presenting before one year of age (3). Inherited in an autosomal recessive manner, FHLH is characterized by the presence of germline homozygous or compound heterozygous loss-of-function (LOF) mutations in a defined set of FHLH-related genes consisting of *PRF1, UNC13D, STX11*, and *STXBP2*, which comprise FHLH subtypes 2-5, respectively. Related immunologic disorders characterized by germline biallelic LOF mutations in genes such as *LYST, AP3B1*, and *RAB27A* similarly underlie the development of HLH. Each of these genes is essential to the cytolytic activity of cytotoxic T-lymphocytes (CD8+ T-cells, hereafter referred to as CTLs) and NK cells (2). In contrast, secondary HLH has historically been diagnosed in patients without a clear genetic predisposition to immune dysregulation. Most commonly presenting in later childhood or adulthood, these patients develop hyperinflammation following exposure to a strong immunogenic stimulus such as an infection or malignancy (4). Many reports have demonstrated that homozygous LOF variants affecting FHLH genes result in early disease presentation, sometimes without an identifiable trigger (5). However, patients harboring less damaging variants, such as missense alterations that impair but do not eliminate protein expression or function, may also develop disease at a later age or in response to a more significant immunogenic stimulus (5). Thus, familial and secondary HLH are often challenging to differentiate in the absence of germline genetic testing.

In addition to the classic monogenic model of autosomal recessive inheritance as a cause for FHLH, there is increasing evidence in favor of a mechanism mediated by digenic inheritance (DI), defined broadly as germline genetic variation at two distinct loci that cooperate to mediate disease (6). Practically, this definition is more nuanced, as there is a spectrum of the extent to which two co-inherited variants interact. As a result, it has been proposed that DI be subclassified into pseudo-DI and true DI. In pseudo-DI, the inheritance of one pathogenic variant is itself sufficient to cause disease. However, the phenotype may be modified by co-inheritance of a second pathogenic variant that when present, enhances disease severity. In this scenario, the disease may be considered monogenic but with a variable phenotype determined by the presence of another variant that functions as a genetic modifier. True DI, in contrast, occurs when mutations in two different genes are required for disease to occur, representing oligogenic inheritance (7). In this review, we discuss the functions of genes associated with FHLH and related immunologic disorders and describe the consequences resulting from LOF mutations in those genes, providing a framework for considering how LOF mutations in two distinct genes within the same functional pathway may cooperate to mediate disease. We then review the clinical data suggesting potential DI and consider areas in which additional research is necessary to better understand the functional implications of this genetic mechanism.

BIOLOGY OF THE DEGRANULATION PATHWAY

CTLs and NK cells respond to and eliminate infected or malignant cells *via* the release of cytotoxic granules (CGs) at the immunological synapse (IS) formed at the site of target cell engagement (8). These CGs contain serine proteases, known as granzymes, which enter the target cell and induce caspase-dependent and -independent apoptosis (9). Successful target cell killing *via* the degranulation pathway requires the coordinated activity of numerous proteins involved in granule biogenesis, trafficking, and exocytosis (**Figure 1**). Homozygous or compound heterozygous LOF mutations in any one of these respective genes underlie HLH and related immune dysregulation syndromes by impeding the ability to terminate the immune response (**Table 1**). Here, we briefly review the molecular and biochemical functions of these genes, focusing on the consequences of LOF mutations. For more detailed information about protein function, we refer the reader to several excellent reviews on this topic (9–12).

Biogenesis

CGs are specialized secretory lysosomes that are assembled in the cytoplasm of CTLs and NK cells. Granzymes and other granule contents are trafficked from the Golgi network to the core of these lysosomes, where an acidic microenvironment maintains the cytotoxic proteins in an inactive state prior to their release at the IS. Two key effectors of proper CG biogenesis are the lysosomal trafficking regulator (LYST) and the clathrin adaptor protein 3 (AP-3) complex (9).

Biallelic LOF mutations in *LYST* underlie Chediak-Higashi syndrome (CHS), a rare inherited immune disorder in which patients can develop the signs and symptoms of HLH. The clinical features of CHS, including oculocutaneous albinism, prolonged bleeding, neurodegeneration, and immune dysregulation, are all attributable to widespread secretory granule dysfunction in multiple cell types. In particular, the impact of these mutations on immune cell function predisposes to hyperinflammation, which is the primary cause of mortality for patients with CHS (13). Functionally, CTLs and NK cells from patients with CHS are characterized by the presence of fewer but significantly enlarged CGs relative to wild-type (WT) cells (9). In WT CTLs, CGs are undetectable prior to exposure to an immune stimulus. Upon CTL activation, cytotoxic protein expression is rapidly upregulated, concomitant with the assembly of numerous, small CGs in the cytoplasm (14). In contrast, in CHS CTLs, CGs initially form identically to those in WT CTLs, but at the later stages of CTL activation, can be seen forming giant intracellular structures (14).

LYST-deficient CTLs and NK cells display impaired target cell killing, suggesting that these giant CGs are unable to successfully release cytotoxic proteins at the IS. Gil-Krzewska et al. studied

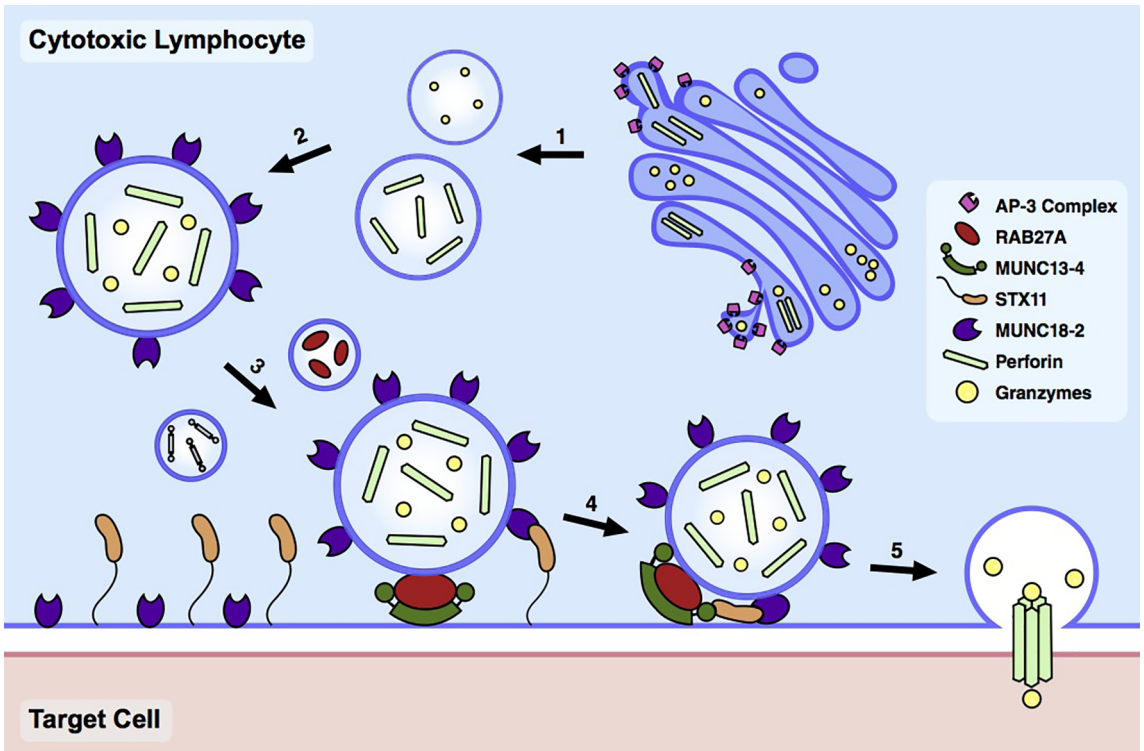

FIGURE 1 | Cytotoxic lymphocyte degranulation pathway. Upon target cell engagement, the degranulation pathway in cytotoxic lymphocytes undergoes a series of coordinated steps consisting of (1-2) cytotoxic granule biogenesis, (3-4) docking and priming, and (5) fusion and pore formation.

TABLE 1 | Degranulation Pathway Genes in HLH and Related Immune Dysregulation Syndromes.

Disease	Gene	Protein	Primary Function
Chediak-Higashi Syndrome	*LYST*	LYST	Biogenesis
Hermansky-Pudlak Syndrome	*AP3B1*	β3A subunit of AP-3 complex	
Griscelli Syndrome Type 2	*RAB27A*	RAB27A	Docking
Familial HLH Type 3	*UNC13D*	MUNC13-4	Priming
Familial HLH Type 4	*STX11*	STX11	Fusion
Familial HLH Type 5	*STXBP2*	MUNC18-2	
Familial HLH Type 2	*PRF1*	Perforin	Delivery

Summary of the degranulation pathway genes and associated proteins underlying FHLH and related immune dysregulation syndromes.

NK cells from patients with CHS carrying mutations in the ARM/HEAT (armadillo/huntingin/elongation factor 3, protein phosphatase 2A, TOR1) domain of *LYST*, a functional domain involved in vesicular trafficking. These cells had fewer and larger CGs relative to WT NK cells. While these large CGs appropriately localized to the IS following target cell engagement, they were unable to fuse with the plasma membrane, thereby preventing the release of cytotoxic proteins toward the target cell (15). To further study the functional consequences of these ARM/HEAT domain mutations, the same authors used CRISPR-Cas9 genome editing to mutate *LYST* in a human NK cell line. As observed in the patient cells, these knockout cells had a small number of large CGs. In order for CGs to fuse with the plasma membrane to release their contents, they must traverse a network of cortical actin that accumulates at the IS. These authors demonstrated that the formation of this network was identical in WT and knockout cells, however the enlarged granules from the *LYST*-deficient cells were excluded from the small openings within the actin meshwork, impeding their localization to the plasma membrane. Treatment of these cells with compounds that interfere with actin polymerization potentiated degranulation and restored cytotoxicity, indicating that CGs in *LYST*-deficient cells retain functionality but are prevented from reaching the cell surface as a result of their excessively large size (16).

The AP-3 protein complex is involved in the formation of clathrin-coated vesicles that traffic to and from lysosome-related organelles (17), a process that is essential for CG biogenesis. Hermansky-Pudlak syndrome (HPS) is a collection of ten autosomal recessive immune disorders mediated by LOF

mutations in the subunits of this protein complex. HPS shares a number of clinical features with CHS, including albinism, a bleeding diathesis, and immune dysregulation, reflecting a shared underlying pathophysiology related to secretory lysosome dysfunction. In particular, the immune dysregulation in patients with HPS type 2 (HPS2), caused by biallelic LOF mutations in the β3A subunit (*AP3B1*) of the AP-3 complex, has been associated with the development of HLH (18).

Cells from patients with HPS2 have decreased protein expression of all AP-3 complex subunits relative to WT cells, as the β3A subunit is thought to stabilize this multi-protein complex. LOF mutations in this subunit alter the conformation of the complex and increase its susceptibility to proteolytic degradation (19). As a result, cells from patients with HPS2 lack the function of the entire AP-3 complex. While LYST regulates the size of CGs, the AP-3 complex is necessary for the appropriate localization of key lysosomal proteins to these granules, which are in turn required for their function. Specifically, this complex mediates the shuttling of proteins from the Golgi network to the lysosome (9). Studies using fibroblasts (20), CTLs (21), and NK cells (22) have consistently demonstrated increased mis-localization of lysosomal proteins to the plasma membrane in cells from patients with HPS2.

Docking and Priming

Following their transport along microtubule pathways to the IS, CGs must dock at the plasma membrane and undergo a priming step prior to their fusion with the membrane and subsequent exocytosis of their contents. Critical mediators of these processes include RAB27A and MUNC13-4, respectively (9).

Griscelli syndrome type 2 (GS2) is an autosomal recessive immune disorder characterized by biallelic LOF mutations in *RAB27A*, a gene encoding a small GTPase. As in CHS and HPS2, patients with GS2 present with albinism, neurologic sequelae, and immune dysregulation frequently associated with the development of HLH (23). In regards to the latter, CTLs from patients with GS2 produce CGs with appropriate contents, but these granules are unable to be released *via* exocytosis, correlating with impaired cytotoxicity (24).

Using CTLs from *ashen* mice, a murine model of human GS2 in which animals lack *Rab27a* expression, Stinchcombe et al. studied the mechanistic basis for the cytotoxicity defect in this disease. The authors demonstrated that upon target cell engagement, CGs in RAB27A-deficient CTLs appropriately migrated along microtubules to re-localize to the IS. However, using electron microscopy to obtain detailed images of the IS, they found that these properly localized granules failed to dock to the membrane, with a subsequent failure to release their contents into the IS (25).

Similar to the phenotype in cells from patients with GS2, CTLs and NK cells from patients with FHLH3 demonstrate impaired cytotoxicity despite appropriate localization of CGs to the IS following cell activation. FHLH3 is characterized by biallelic LOF mutations in *UNC13D*, which encodes the MUNC13-4 protein. Like RAB27A, the vesicular distribution of MUNC13-4 in resting CTLs is largely distinct from that of granzymes. Following cell activation and formation of the IS, vesicles containing RAB27A, vesicles containing MUNC13-4, and CGs co-localize at the IS and fuse into a common vesicular structure (26). While RAB27A mediates the docking of these CGs, MUNC13-4, itself tethered to the membrane *via* a required protein-protein interaction with the small GTPase RhoG (27), is required for the final step prior to granule fusion with the plasma membrane. This final process, known as priming, makes these granules competent for exocytosis (9). As a result, CGs in MUNC13-4-deficient CTLs can be seen docking at the plasma membrane, but failing to undergo exocytic fusion (28).

Much of what is known about the priming function of MUNC13-4 at the IS comes from studies of the role of other MUNC13 family members in regulating neurotransmitter release at neurological synapses. Using total internal reflection fluorescence microscopy (TIRFM) in neuroendocrine cells, it has been shown that following MUNC13-mediated priming, vesicles docked at the plasma membrane have significantly reduced mobility (29). Similarly, in activated MUNC13-4-deficient murine CTLs, the docked CGs are significantly more mobile than those in WT CTLs, and this phenotype can be rescued with ectopic expression of MUNC13-4 (30).

Fusion

After docking and priming, CGs are finally able to fuse with the plasma membrane, enabling the release of their cytotoxic contents across the IS toward the target cell. This fusion process is mediated by the activity of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), a family of proteins that are ubiquitous through the immune system, where they function to mediate the fusion of docked vesicles with their target membranes. While the requirement for specific SNARE proteins differs according to cell type, the mechanism is shared for all vesicular fusion events. Specifically, SNARE proteins on the vesicular membrane lock together with SNAREs on the target cell membrane to form a protein bridge between the two structures. This interaction then pulls the two compartments into close proximity, and a subsequent conformational change in the SNARE protein complex generates a force that is sufficient to fuse the lipid bilayers of the two membrane compartments (31). These SNARE protein interactions are further regulated by the activity of SNARE accessory proteins, as binding to these accessory proteins is required for SNARE-mediated vesicle fusion with the plasma membrane (9).

LOF mutations in the genes *STX11*, encoding the SNARE protein syntaxin 11, and *STXBP2*, encoding the SNARE accessory protein MUNC18-2, underlie FHLH4 (32) and FHLH5 (33), respectively. Like patients with FHLH3, patients harboring biallelic *STX11* and *STXBP2* mutations demonstrate impaired CTL and NK cell degranulation with an associated cytotoxicity defect, despite appropriate mobilization of CGs to the IS following cell activation (33, 34). Analyzing lymphocytes from patients with FHLH4 and FHLH5, Côte et al. highlighted the importance of interactions between these two proteins. Specifically, in cells lacking MUNC18-2, they found markedly reduced expression of STX11, suggesting that MUNC18-2 is required for STX11 stabilization. The converse was not true, as

MUNC18-2 expression was the same in WT and FHLH4 lymphocytes (33). This finding is further supported by work from Dieckmann et al. demonstrating a role for MUNC18-2 as a chaperone of STX11 at the plasma membrane. Using healthy human CTLs, they demonstrated that MUNC18-2 localizes primarily to CGs, traveling with them to the IS upon target cell engagement. In contrast, STX11 was found primarily at the plasma membrane, and became concentrated at the IS after cell activation. While this localization of MUNC18-2 was unchanged in the absence of STX11, STX11 was lost from the plasma membrane in cells lacking MUNC18-2 expression (35), suggesting that MUNC18-2 is required both for the stability and the proper subcellular localization of STX11. Intriguingly, in addition to its chaperone capacity, MUNC18-2 may also play a functional role in the fusion of CGs with the plasma membrane. Spessott et al. demonstrated that STX11, while anchored to the plasma membrane, can support the exchange of lipids between vesicular compartments, but cannot independently facilitate the exchange of vesicular contents. With the addition of WT MUNC18-2, but not a mutant incapable of binding to STX11, complete fusion could be induced (36). These data indicate the importance of an intact molecular interaction between STX11 and MUNC18-2 to facilitate CG fusion, thereby underscoring the defective cytotoxicity in cells from patients with either FHLH4 or FHLH5.

Delivery

Once CGs are released from the CTL or NK cell, they traverse the IS to induce target cell apoptosis. While the granzyme contents of the CGs directly mediate apoptosis, their activity is dependent on perforin (*PRF1*), a pore-forming protein that is required for the delivery of granzymes into the target cell cytoplasm (37). The importance of perforin for the cytotoxic activity of CTLs and NK cells is reflected in the severity of the clinical phenotype associated with LOF mutations in *PRF1*, which was the first identified FHLH gene. Patients with biallelic *PRF1* mutations, now known as FHLH2, commonly present early in life with severe immune dysregulation (38).

Given the clinical significance of impaired PRF1 function, there has been considerable interest in understanding how PRF1 mediates granzyme access to the target cell cytoplasm. It is well-established that the pore-forming capabilities of PRF1 are dependent on its oligomerization into a complex that has structural similarity to the membrane attack complex formed in the innate immune system (39). Using human cell lines, Keefe et al. demonstrated that exposing cells to PRF1 induces rapid and profound changes to the plasma membrane, with the formation of membrane blebs and a loss of membrane integrity that facilitates the translocation of cell impermeable dyes into the cytoplasm. Intriguingly, when they tracked the localization of these dyes, they observed that, rather than disseminating into the cytosol, they remained contained within membrane-proximal blebs. Similarly, when cells were incubated in the presence of granzyme B and exposed to PRF1, granzyme B could be found within vesicular structures in the target cell cytoplasm, suggesting that PRF1 mediates entry into target cells *via* an endocytic process (40). These same authors went on to demonstrate that these endocytic structures, termed "gigantosomes", contain both PRF1 and granzymes. Approximately 15 minutes after their formation, these structures rupture due to PRF1-induced pore formation in the endosome membrane. This then releases endosomal contents, thereby exposing the target cell cytoplasm to apoptosis-inducing granzymes (41).

A THRESHOLD MODEL OF GENETIC PREDISPOSITION TO HLH

FHLH and related immune disorders associated with an HLH phenotype have traditionally been defined as autosomal recessive diseases characterized by germline biallelic LOF mutations in one of the degranulation pathway genes, which in turn confer defective lymphocyte cytotoxicity. This is in contrast to secondary HLH, which is diagnosed in patients meeting the clinical diagnostic criteria for HLH in the absence of a clear genetic predisposition. However, there is increasing evidence that this dichotomous definition is likely an oversimplification, as it does not adequately address the complex interactions between genetic predisposition and environmental influence. A more comprehensive model considers a threshold for disease development, in which severe inherited dysfunction in the degranulation pathway is sufficient to mediate disease with little or no identifiable environmental trigger, while more subtle insults to pathway function require a profound immunogenic stimulus to induce hyperinflammation (42–44). This model suggests that a multitude of genetic mechanisms may contribute to the risk of developing HLH by mediating some degree of underlying dysfunction in the lymphocyte degranulation pathway (**Figure 2**).

Even amongst those patients with a classic autosomal recessive pattern of inheritance, clinical and experimental data suggest that LOF mutations in different degranulation pathway genes have non-equivalent consequences, with loss of some proteins resulting in a more severe phenotype than others. In a cohort of patients with complete LOF of *PRF1, RAB27A*, or *STX11*, it was found that the severity of disease differed as a function of the involved gene. At one extreme, patients with *PRF1* mutations presented with severe, early-onset disease, while those with *STX11* mutations had milder disease and presented at a later age (45). These clinical data are recapitulated in murine models of degranulation pathway dysfunction. When Jessen et al. infected multiple HLH-prone murine models with lymphocytic choriomeningitis virus (LCMV) to stimulate the development of HLH, they observed a spectrum of disease severity that correlated with the extent of the cytotoxicity defect conferred by the respective gene knockout. Based on this analysis, they proposed a hierarchy of degranulation pathway genes, with mutations in *Ap3b1* conferring the mildest phenotype followed by *Lyst, Stx11, Rab27a*, and finally *Prf1*. Interestingly, when they analyzed a cohort of patients with complete LOF of these same genes, they found the same hierarchy reflected in the average age of disease onset (46), suggesting that even amongst patients with biallelic LOF mutations, a threshold model of disease may apply.

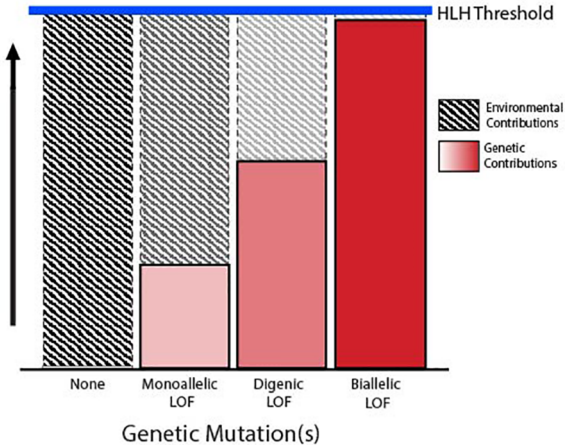

FIGURE 2 | Threshold model of HLH. Schematic depicting the inverse relationship between the degree of degranulation pathway dysfunction conferred by germline variants and the environmental trigger required to reach the threshold for development of HLH.

Beyond this autosomal recessive mechanism, clinical evidence also suggests that inheritance of a single heterozygous mutation in a degranulation pathway gene may be sufficient to predispose to HLH. Zhang et al. reported on two unrelated teenagers with HLH who were found to have the same heterozygous missense mutation in *RAB27A*. When this mutation was expressed in an NK cell line, the authors found impaired degranulation and cytotoxic activity as well as decreased capacity for binding to MUNC13-4. Interestingly, clinical NK cell studies in one of these patients similarly revealed impaired NK cell function. Her father was found to be the carrier of the mutation and had a comparable degree of NK cell dysfunction but was otherwise healthy (47), suggesting that this *RAB27A* mutation may predispose to HLH *via* cooperation with other currently unknown genetic modifiers and environmental triggers. Heterozygous mutations in *RAB27A* and *STXBP2* have also been shown to mediate degranulation pathway dysfunction by acting in a dominant-negative fashion to impair CG docking and fusion (47, 48). Similarly, monoallelic mutations in *STXBP2* and *LYST* have been reported to occur with significantly greater frequency in patients with systemic juvenile idiopathic arthritis (sJIA) who develop clinical manifestations of HLH relative to those patients with sJIA who do not show signs of hyperinflammation (49). In another cohort of 175 patients with adult-onset HLH, 25 were found to have single heterozygous missense or splice-site mutations in *PRF1, MUNC13-4*, or *STXBP2*. Out of the 14 of these patients who had NK cell studies performed, nine had low or absent function. Interestingly, 12 carried the A91V allele of *PRF1*, an allele that is found at a frequency of up to 4-7% in healthy control populations (50). However, functional studies have demonstrated that expression of this allele on an otherwise WT background does confer cytotoxic dysfunction (51), and that this allele is significantly more prevalent in patients with HLH relative to healthy controls (52), suggesting that it may be a hypomorphic allele that predisposes to HLH.

Together, these data suggest that even partial genetic impairments of the degranulation pathway can be sufficient to predispose to HLH in cooperation with an adequately strong environmental stimulus and/or other genetic modifiers. Similarly, cumulative monoallelic "hits" affecting different genes within the degranulation pathway might also negatively impact immune cell function and increase the risk for HLH. In this way, patients who are doubly heterozygous for mutations in two distinct degranulation pathway genes, also known as DI, could manifest an HLH phenotype that is intermediate between one resulting from a biallelic LOF *versus* a single heterozygous LOF mutation.

At least one functional study suggests that multiple genetic hits to the degranulation pathway may contribute to a predisposition to HLH. Sepulveda et al. used murine models to study the functional consequences of combinations of heterozygous LOF in *Rab27a/Stx11, Rab27a/Prf1*, and *Rab27a/Stx11/Prf1*. In each of these mouse strains, the respective protein expression was reduced by 50%, concomitant with reduced cytotoxicity and manifestations of HLH, though the phenotypic severity differed across the combinations. Mice with the *Rab27a/Prf1* combination had a more severe phenotype than did mice with the *Rab27a/Stx11* combination, while mice with LOF of all three had the most profound NK cell dysfunction and disease severity (53), suggesting that cumulative effects on the degranulation pathway mediate hyperinflammation.

Next, we summarize available clinical data describing patients with DI of degranulation pathway genes, providing when available information regarding age of HLH onset and functional studies as correlates of the degree of degranulation pathway dysfunction.

CASE REPORTS AND COHORT STUDIES

At least two case reports have described patients with HLH who were found to have monoallelic variants affecting two distinct degranulation pathway genes. One report describes a 32-year-old male who presented with fever, rash, joint pain, nausea, and vomiting and was subsequently diagnosed with adult-onset Still's disease (54). After initial management with prednisone and antibiotics, he re-presented with fever, splenomegaly, hyperferritinemia, and hypertriglyceridemia, fulfilling four HLH diagnostic criteria. Despite further treatment with prednisone, the patient subsequently died of progressive multi-organ dysfunction. Later genetic evaluation revealed a heterozygous variant in *UNC13D* and another in *AP3B1*, though no NK cell functional studies were performed.

A second report presents the case of a 30-year-old female with chronic active Epstein Barr virus (CAEBV) infection (55). The patient fulfilled diagnostic criteria for HLH, including fever, pancytopenia, hypofibrinogenemia, hyperferritinemia, elevated soluble CD25, decreased NK cell function, and impaired CD107a mobilization, an indicator of degranulation pathway dysfunction.

Treatment with etoposide and dexamethasone per the HLH-2004 protocol successfully ameliorated her hyperinflammation. Whole exome sequencing (WES) identified a heterozygous variant in *STXBP2* and another in *LYST*. Intriguingly, the patient's mother also carried these two variants and had severely impaired NK cell function, but had never developed HLH, again suggesting that additional environmental and genetic factors may have cooperated with underlying degranulation pathway dysfunction to trigger HLH in this patient. Specifically, while the patient had a long history of CAEBV, the mother was noted to be negative for EBV, which is a well-established trigger of HLH in patients with underlying genetic predisposition (56). Through whole exome sequencing (WES), the patient was also found to carry three additional heterozygous variants affecting *LRBA* (encoding LPS-responsive beige-like anchor protein), *AIRE* (encoding autoimmune regulator protein), and *IRF8* (encoding interferon regulator factor 8). Importantly, all three of these genes have demonstrated functions in immune regulation (57–59), and the latter two variants were inherited from the patient's father, suggesting that they may have modified the patient's risk for developing HLH in response to an adequate immunogenic stimulus. These data underscore the importance of WES for the identification of potential genetic modifiers of underlying degranulation pathway dysfunction.

In addition to these reports, multiple groups have performed retrospective studies of cohorts of patients with HLH who underwent clinical genetic testing, most commonly *via* targeted sequencing of HLH-associated genes. Several of these studies have identified a small number of patients with heterozygous variants in two different degranulation pathway genes. In one cohort of 24 patients who were heterozygous for the A91V (c.272C>T) allele of *PRF1*, three were found to also carry heterozygous variants in *UNC13D*. Two of the three had confirmed NK cell dysfunction and presented with HLH prior to one year of age (60). Analysis of 94 Vietnamese patients identified a child presenting with HLH prior to one year of age who had single variants in both *UNC13D* and *STX11* (61). In a cohort of 14 patients with sJIA and HLH, one was found have variants in *LYST* and *STXBP2*, and had absent NK cell function and the presence of hemophagocytosis on bone marrow biopsy (49). In two cohorts of adult Chinese patients, two patients with DI of degranulation pathway variants were identified. One was found to have heterozygous variants in both *STX11* and *LYST* (62). Another had variants in *UNC13D* and *LYST*, and corresponding functional studies showed significantly impaired NK cell function and CD107a mobilization (63). Additional cohort studies presenting patients with DI of degranulation pathway genes are summarized in **Table 2**.

In the largest study to date directly evaluating the plausibility of DI in HLH, Zhang et al. analyzed targeted sequencing data from 2,701 patients with HLH and identified 28 with heterozygous variants in two different degranulation pathway genes. Twenty-one of these patients had variants in *PRF1* plus another gene, while the remaining seven had variants in two other genes in the degranulation pathway. These seven tended to have an earlier age of onset, comparable to that of patients with classic biallelic LOF mutations. Of the four who had corresponding functional studies, three had impaired CD107a mobilization. Based on these data, the authors conclude that cooperation between two distinct variants likely mediates immune dysregulation that is sufficient to cause HLH, though they acknowledge the caveat of incomplete functional data (64).

Finally, in a cohort of 48 pediatric patients with HLH who underwent WES, Chinn et al. identified heterozygous variants in two degranulation pathway genes in seven (14.5%) patients. To determine the significance of these findings, the authors compared the genotypes observed in their patient cohort to genotypes present in the Baylor-Hopkins CMG database. Intriguingly, they concluded that these combinations were unlikely to occur more commonly in patients with HLH than in the general population, and based on this result, cautioned that other studies reporting on the clinical significance of DI should be interpreted carefully (65).

The data from these case reports and cohort studies are summarized in **Table 2**. To enable comparisons between these studies, we used ClinVar (67) and CADD scores (68), reported as PHRED-scaled scores, to determine the significance of the reported variants as known at the time of publication of this article.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Here, we review the consequences of LOF mutations in genes encoding several key components of the lymphocyte degranulation pathway, providing a mechanistic basis for interpreting the clinical significance of variants in one or more of these genes. Additionally, we summarize the clinical and experimental data that support revisiting the historical division of HLH into its familial and secondary forms. These data instead favor a more comprehensive model that takes into account both genetic and environmental factors, resulting in a threshold over which the combined effect of these two factors can lead to the development of HLH in the context of an appropriately strong antigenic stimulus. In support of this model, we present an analysis of 44 patients with HLH lacking the classic biallelic LOF mutations but found instead to harbor heterozygous variants in two different genes within the degranulation pathway (**Table 2**).

While the clinical data presented here suggest the plausibility of DI in HLH, several caveats limit the interpretation of these data. First, of the variants identified in these studies that have information available in the ClinVar database, 33% are reported as "benign" or "likely benign" and 58% have "uncertain significance" or "conflicting interpretations of pathogenicity". Without corresponding functional data, it is not possible to determine their true impact on protein expression or activity. In addition, clinical NK cell function and CD107a mobilization studies are lacking for many of these patients, further complicating the interpretation of any putative functional impacts of the reported genotypes. Second, most of the studies summarized here relied on targeted sequencing of HLH-associated genes rather than on WES, precluding the ability to identify other potential genetic causes of immune dysregulation.

TABLE 2 | Summary of Clinical Data of Patients with DI of Degranulation Pathway Gene Mutations.

Age at Diagnosis	Nucleotide Change	Protein Change	ClinVar Significance	PHRED Score	Reference
32 years	UNC13D c.1232G>A	p.Arg411Gln	Likely benign	22.1	(54)
	AB3B1 c.1075A>G	p.Thr359Ala	Uncertain Significance	25.6	
30 years	STXBP2 c.592A>C	p.Thr198Pro	–	24.3	(55)
	LYST c.830A>T	p.His277Leu	–	16.29	
2 months	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	(60)
	UNC13D c.I825C>T	p.Gln609X	–	5.396	
7 months	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2346-2349del4	p.Arg782fs	Pathogenic	–	
28 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.182A>G	p.Tyr61Cys	–	–	
1 year	UNC13D c.965_967>68bp	p.Ala318X	–	–	(61)
	STX11 c.122T>C	p.Leu41Pro	–	–	
4 years	LYST c.1940T>G	p.Leu647Arg	Uncertain significance	24.2	(49)
	STXBP2 del7705108	–	–	–	
–	LYST c.7994A>G	p.Asp2665Gly	Uncertain significance	23.6	(62)
	STX11 c.842T>G	p.Phe281Cys	Uncertain significance	15.34	
18 years	LYST c.11268-5delT	–	Benign/Likely benign	–	(63)
	UNC13D c.1120C>A	p.Pro374Thr	–	–	
3 months	PRF1 c.1310C>T	p.Ala437Val	Conflicting IOP	25	(64)
	UNC13D c.169G>T	p.Glu57X	–	–	
9 months	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2709+6G>T	–	Benign/Likely benign	–	
11 months	PRF1 c.992C>T	p.Ser331Leu	Uncertain significance	23.8	
	UNC13D c.1232G>A	p.Arg411Gln	Likely benign	22.1	
2.25 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.227C>T	p.Thr76Met	Conflicting IOP	4.913	
3 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.869C>T	p.Ser290Leu	Uncertain significance	0.059	
3 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2243C>T	p.Ala748Val	Uncertain significance	18.37	
5 years	PRF1 c.1229G>A	p.Arg410Gln	Conflicting IOP	19.5	
	UNC13D c.1036G>A	p.Asp346Asn	–	–	
8 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.3160A>G	p.Ile1054Val	Uncertain significance	10.51	
9 years	PRF1 c.10C>T	p.Arg4Cys	Conflicting IOP	0.163	
	UNC13D c.3232G>C	p.Ala1078Pro	–	23.2	
9 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
10 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
12 years	PRF1 c.50delT	p.Leu17fs	Pathogenic	–	
	UNC13D c.1579C>T	p.Arg527Trp	Benign/Likely benign	21.3	
13 years	PRF1 c.445G>A	p.Gly149Ser	Pathogenic	23.7	
	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
13 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
5 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	STXBP2 c.1034C>T	p.Thr345Met	Benign/Likely benign	25.6	
10 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	STXBP2 c.1034C>T	p.Thr345Met	Benign/Likely benign	25.6	
16 years	PRF1 c.655T>A	p.Tyr219Asn	–	28.6	
	STXBP2 c.1034C>T	p.Thr345Met	Benign/Likely benign	25.6	
21 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	
	STXBP2 c.1586G>C	p.Arg529Pro	Conflicting IOP	26	
24 years	PRF1 c.50delT	p.Leu17fs	Pathogenic	–	
	STXBP2 1459G>A	p.Val487Met	Likely benign	23	
2 months	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
	STXBP2 c.911C>T	p.Thr304Met	Uncertain Significance	26.2	
5 months	UNC13D c.1389+1G>A	–	Pathogenic	–	
	STXBP2 c.1782*12G>A	–	–	–	
8 months	UNC13D c.2828A>G	p.Asn943Ser	Likely benign	25.7	
	STXBP2 1782*12G>A	–	–	–	
1 year	UNC13D c.2828A>G	p.Asn943Ser	Likely benign	25.7	

(Continued)

TABLE 2 | Continued

Age at Diagnosis	Nucleotide Change	Protein Change	ClinVar Significance	PHRED Score	Reference
	STXBP2 c.715C>T	p.Pro239Ser	–	23.9	
2 months	UNC13D c.2030T>C	p.Ile677Thr	–	26.4	
	STX11 c.221C>T	p.Thr74Met	Conflicting IOP	26.2	
14 years	STXBP2 c.568C>T	p.Arg190Cys	Conflicting IOP	31	
	STX11 c.9C>A	p.Asp3Glu	Uncertain significance	23.8	
5 years	STXBP2 c.1034C>T	p.Thr345Met	Benign/Likely benign	25.6	
	RAB27A c.295T>G	p.Phe99Val	–	28.7	
1 year	LYST c.11268-5delT	–	Benign/Likely benign	–	(65)
	STXBP2 c.1474G>A	p.Asp492Asn	–	29.7	
2 years	LYST c.4732G>A	p.Ala1578Thr	–	–	
	UNC13D c.2341G>A	p.Val781Ile	Conflicting IOP	0.764	
7 years	LYST c.11268-5delT	–	Benign/Likely benign	–	
	UNC13D c.2917A>G	p.Lys973Glu	Uncertain significance	23.1	
15 years	LYST c.10688C>T	p.Ser3563Leu	–	32	
	PRF1 c.655T>A	p.Tyr219Asn	–	28.6	
16 years	LYST c.11268-5delT	–	Benign/Likely benign	–	
	UNC13D c.2896C>T	p.Arg966Trp	Benign/Likely benign	26.3	
17 years	LYST c.10800+4G>T	–	Conflicting IOP	–	
	STX11 c.9C>A	p.Asp3Glu	Uncertain significance	23.8	
–	LYST c.4265C>T	p.Ala1422Val	Uncertain significance	22.2	
	RAB27A c.418C>G	p.Gln140Glu	Conflicting IOP	18.45	
1 year	PRF1 1349C>T	p.Thr450Met	Pathogenic	23.5	(66)
	AP3B1 c.1321A>G	p.Ile441Val	–	–	
25 years	PRF1 c.272C>T	p.Ala91Val	Conflicting IOP	24.8	(50)
	STXBP2 c.795-4C>T	–	Benign/Likely benign	–	

Summary of age at HLH onset and identified degranulation pathway variants for 44 patients reported in published case reports and cohort studies. "-" indicates information not available, IOP – interpretations of pathogenicity.

Finally, as noted by Chinn et al. (65), many of the reported digenic combinations are also present at some frequency in healthy populations, confounding the attribution of these genotypes to the development of HLH in these patients. Taken together, the data presented here suggest that co-inheritance of variants in degranulation pathway genes does occur in a subset of patients who develop HLH. However, more comprehensive studies consisting of WES in combination with functional and biochemical analyses of the associated variants and correlation with environmental triggers will be required to better understand the impact of these variant combinations on predisposition to HLH and the extent to which these combinations manifest clinically as pseudo-DI or true DI.

AUTHOR CONTRIBUTIONS

ES and LM wrote the manuscript. MH and KN reviewed and edited the manuscript. All authors approved the submitted version.

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Autoimmune Cytopenias and Dysregulated Immunophenotype Act as Warning Signs of Inborn Errors of Immunity: Results From a Prospective Study

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Inborn errors of immunity (IEI) are genetic disorders characterized by a wide spectrum of clinical manifestations, ranging from increased susceptibility to infections to significant immune dysregulation. Among these, primary immune regulatory disorders (PIRDs) are mainly presenting with autoimmune manifestations, and autoimmune cytopenias (AICs) can be the first clinical sign. Significantly, AICs in patients with IEI often fail to respond to first-line therapy. In pediatric patients, autoimmune cytopenias can be red flags for IEI. However, for these cases precise indicators or parameters useful to suspect and screen for a hidden congenital immune defect are lacking. Therefore, we focused on chronic/refractory AIC patients to perform an extensive clinical evaluation and multiparametric flow cytometry analysis to select patients in whom PIRD was strongly suspected as candidates for genetic analysis. Key IEI-associated alterations causative of STAT3 GOF disease, IKAROS haploinsufficiency, activated PI3Kδ syndrome (APDS), Kabuki syndrome and autoimmune lymphoproliferative syndrome (ALPS) were identified. In this scenario, a dysregulated immunophenotype acted as a potential screening tool for an early IEI diagnosis, pivotal for appropriate clinical management and for the identification of new therapeutic targets.

Keywords: autoimmune cytopenia, autoimmune thrombocytopenia, autoimmune hemolytic anemia, autoimmune neutropenia, Evans syndrome, immunophenotyping, primary immune regulatory disorder (PIRD), inborn errors of immunity (IEIs)

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INTRODUCTION

Inborn errors of immunity (IEI) are an expanding universe of disorders, not only characterized by an infectious diathesis but also displaying a wide variety of other clinical features (1). Primary Immune Regulatory Disorders (PIRDs) are a relevant subgroup of IEI that is particularly characterized by autoimmune manifestations (2, 3). The number of genetic defects belonging to this category has strikingly expanded over time (4), and atypical manifestations of known PIRDs have progressively been unveiled (5).

In this dynamic setting, target organs of the autoimmune process may be diverse, but autoimmune cytopenias (AICs) undoubtedly play a leading role (6–8). Indeed, the relative risk of AIC appears to be at least 120 times higher in patients with IEI, compared to the general population, and increases up to 830 times if we consider autoimmune hemolytic anemia (AIHA) alone (6). Moreover, the combination of AIHA and immune thrombocytopenia (ITP) is often the clinical presentation of a PIRD (9–11), and potentially bears a genetic explanation in 65% of cases (12). Indeed, some specific immunological alterations, if accompanied with AIHA, ITP, autoimmune neutropenia (AIN), or their combinations (Evans syndrome, ES) could be significant red flags for an associated IEI (13). These include both humoral and cell-mediated immune defects, like reduced serum immunoglobulin levels and low T cell counts (3, 12, 14, 15), while only scant evidence regarding deeper immunological studies in AICs is available (16).

Regarding treatment, AICs in patients with IEI often fail to respond to first-line therapy, and the best management for refractory AICs still needs to be fully elucidated (17–20). Intravenous immunoglobulins (IVIG) and immunosuppressants are, in some cases, effective (17–19); interestingly, immunomodulatory drugs may significantly attenuate immunological alterations in PIRDs – as seen in ALPS (21, 22) – while rituximab can lead to a persistent hypogammaglobulinemia and potentially unmask an underlying genetic defect (23). Importantly, attaining a definitive molecular diagnosis might open new targeted therapeutic options, as seen in LRBA and CTLA-4 deficiencies as well as in other PIRDs (20, 24–26).

In this context, we sought to investigate the immunological and genetic background of pediatric patients affected by refractory mono- or multi-lineage AICs, eventually associated with additional signs of immune dysregulation. We applied extensive multiparametric flow cytometry, an already established tool in detecting and monitoring IEI (27, 28), to lymphocyte phenotyping on AIC background, in order to select patients in whom PIRD was suspected, and to direct next-generation sequencing (NGS) analysis. Immune phenotyping acted as a potential screening tool for an underlying IEI, thus permitting an early molecular diagnosis and a specific treatment.

METHODS

Patient Selection and Data Collection

This prospective study included 30 pediatric and adolescent - young adult (AYA) patients (median age 8,5 years, range 1-24 years) referred to A. Meyer Children Hospital Oncology-Hematology Department for mono- or multilineage AIC, defined by immunological evaluation and/or differential diagnosis with other hematologic causes (i.e. bone marrow failure or malignancies). We recruited patients presenting with: chronic refractory ITP and/or AIHA (>12 months) requiring at least a second-line treatment; and/or AIN not self-resolving (>12 months).

According to the European Hematology Association (EHA) and the Intercontinental Childhood ITP Study (ICIS) working group criteria, ITP was defined by blood platelet count $<100x10^9$/l on two separate measurements (29); AIHA by Hb level <11 g/dl and at least one hemolysis criteria among the following: reticulocytosis $>120x10^9$/l, total bilirubin >1 mg/dl, and haptoglobin <10 mg/dl (30). AIN was defined as neutrophil counts $<1,5x10^9$/l on two separate measurements, after excluding other secondary causes (31). Patients' classification into two groups, isolated AIC (AIC-alone) and AIC with strong suspicion of IEI (AIC-sIEI), was based on clinical signs of immune dysregulation and/or immunodeficiency, defined as: hypogammaglobulinemia, auto/hyper-inflammation, organ-specific autoimmunity, splenomegaly, lymphadenopathy, lymphoid malignancies and/or recurrent or opportunistic infections. Eventual family history of immune disorders was considered as inclusion criteria.

First-line therapy for cytopenias included intravenous immunoglobulin (IVIG) and/or corticosteroids according to local practice; therefore, refractory ITP and AIHA were treated with second- and third-line therapy (mycophenolate mofetil, MMF; sirolimus; rituximab; eltrombopag). Due to the severity of their clinical condition at time of referral, a few patients directly underwent second- or third-line therapy. To assess response to conventional therapy, the following criteria were used: for ITP, a platelet count $>30x10^9$/l with at least a two-fold increase of the pre-treatment count (29). For AIHA, Hb level ≥10 g/dl with an increase of at least 2 g/dl from baseline was considered. A response as defined above lasting at least 2 months would classify a patient as a responder (32).

The study was reviewed and approved by Meyer Hospital Pediatric Ethics Committee, and written informed consent was obtained of all included patients or their parents, according to their age. Data related to patients' clinical and family history was collected (**Supplementary Table 1**). Patients' peripheral blood was analyzed at different time points to perform immunophenotyping (**Figure 1**), whereas genetic analysis was conducted on both patients and their parents in order to better interpret the genetic results.

Immunophenotyping

Peripheral blood (PB) obtained from patients was processed within 24h after collection to perform immunophenotyping. Upon red blood cell lysis with ammonium chloride, cells were stained to identify T, B and NK cell subsets using the monoclonal antibodies listed in **Supplementary Table 2**. Flow cytometry data were collected using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany), and analyzed with Flowlogic Software (v. 7.3, Inivai Technologies, Victoria, Australia). The expression of CD3, CD4, CD8, CD27, CD45RA,

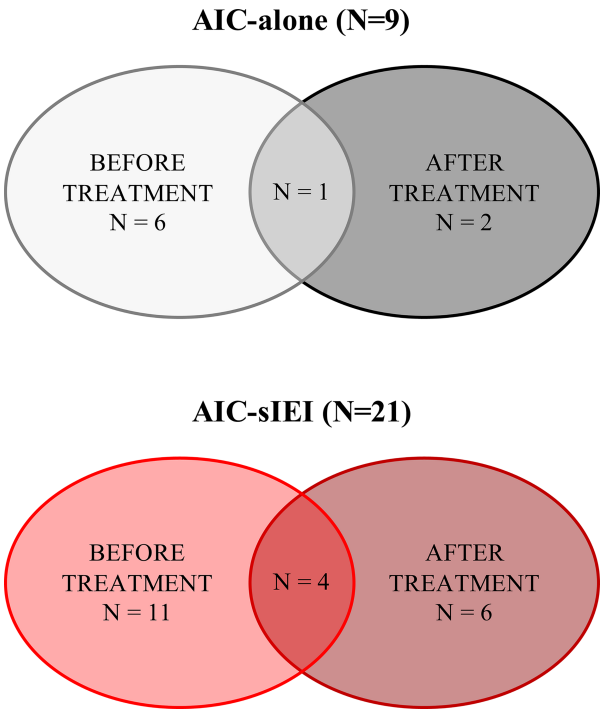

FIGURE 1 | Summary of patients' sample collection. Patients' samples were collected before 2nd-line treatment (IVIG and/or corticosteroids) and/or after 2nd- or 3rd-line treatment, which included MMF, sirolimus, rituximab, and eltrombopag. N, count.

CD31 was used to identify recent thymic emigrants (RTE, CD45RA+CD31+), naïve (CD27+CD45RA+), central memory (CM, CD27+CD45RA-), effector memory (EM, CD27-CD45RA-) and terminally differentiated effector memory T cells (EMRA, CD27-CD45RA+). Treg cells were identified by CD25 and CD127 expression (CD4+CD25+CD127low), and distinguished in naïve (CD45RA+) and memory (CD45RA-) Treg. Double negative T cells (DNT) were identified by CD4 and CD8 expression within the TCRαβ T subset (TCRαβ+CD4-CD8-). The CD19+ B cell subpopulations were defined based on the differential expression of CD27 and IgD into naïve (CD27-IgD+), pre-switched memory (CD27+IgD+) and switched memory (CD27+IgD-). Plasmablasts (IgM-CD38++) and transitional B cells (CD24+CD38+) were evaluated. NK cells were defined based on CD56 expression (CD3-CD56+). A minimum of 20000 events within the lymphocyte population gate were collected, and gating strategy is shown in **Supplementary Table 3**. Absolute cell count was calculated from total lymphocyte numbers obtained by differential blood count.

Genetic Analysis

Genetic testing was performed on the AIC-sIEI group, as patients presenting with isolated AIC (AIC-alone) did not meet the clinical and immunological criteria necessary to suspect an immunodeficit. Genomic DNA (gDNA) was extracted from peripheral blood obtained from patients and their parents using the BioRobot EZ1 Workstation (Qiagen, Milan, Italy) and quantified. Sequencing analysis was performed through target resequencing of 58 immune dysregulation-associated genes (**Supplementary Table 4**) using MiSeq Illumina platform (Illumina, San Diego, USA), or through whole-exome sequencing (WES) according to the protocols indicated. Sequence reads were aligned to the NCBI38/hg38 reference genome using a pipeline based on BWA and Picard, and variants were called using the GATK toolkit. Variants annotation (ANNOVAR tool) and prioritization was performed according to the standard guidelines of the American College of Medical Genetics and Genomics (ACMG) (33), by using a combination of prediction programs (SIFT, PolyPhen, pMUT, Mutation taster, FATHMM score, CADD score) to distinguish potentially damaging variants from those predicted to have neutral effect. Variants that were called less than 5X, off-target, synonymous, or with minor allele frequency (MAF) >1% in the Exome Aggregation Consortium (ExAC, Cambridge, MA http://exac.broadinstitute.org) were eliminated. For WES, data were filtered for a panel of >400 genes published by the International Union of Immunological Societies (IUIS) expert committee on IEI (34).

Statistical Analysis

Analysis of lymphocyte main populations (total lymphocytes, CD3 T cells, CD4 T cells, CD8 T cells, CD19 B cells and CD56 NK cells) count (x10^9/l) was performed using Microsoft Excel (v. 365, Microsoft Corporation, Redmond, USA), and comparisons between the two groups were made using the Student t-test (two-tailed). GraphPad Prism (v. 8.0, San Diego, USA) was used for univariate analysis of CD4, CD8, Treg and B cell subpopulation frequencies, by applying the nonparametric Mann-Whitney test (two-tailed). P values <0.05 were considered significant. Multivariate analysis on T lymphocyte subsets was performed by Principal Component Analysis (PCA), PAleontological STatistics (PAST, v. 4.03, University of Oslo). PCA is a technique for reducing the dimensionality of large datasets, minimizing information loss and increasing interpretability. The majority of the variation of flow cytometric datasets is captured by the 2 most dominant principal components (Component 1 and 2), representing a Cartesian space in which each sample (patient) is allocated. Samples are plotted to visualize similarities and differences. The overlay of the 2D (2 Dimensional) plot of the scores (patients) with the 2D plot of the loadings (combination of cell subsets) allows the identification of the variables that most contribute to the characterization of the single patient.

RESULTS

Patients' Clinical Presentation

We enrolled 30 patients, 21 males (70%) and 9 females (30%) and classified them into two groups: isolated AIC (AIC-alone) and AIC with strong suspicion of IEI (AIC-sIEI) based on the associated other clinical signs of immunodeficiency beyond AIC. Cohort clinical and laboratory features are shown in **Table 1** and **Supplementary Table 1**. The most represented cytopenia

lineages are ITP and AIHA, the latter peculiar to patients with signs of immune defect. In the AIC-sIEI group, splenomegaly and hypogammaglobulinemia are the most frequent clinical signs, and almost all patients with lymphadenopathy (6/7) also presented with splenomegaly.

Imbalance of Naïve and Memory T Lymphocyte Compartments in AIC Patients With Signs of Immune Dysregulation

As we aimed at defining possible congenital immune defects, causative of a wide spectrum of manifestations other than the cytopenia, we performed an extended immunophenotyping on lymphocyte subsets. Regardless of the diagnostic group, 22 patients were investigated before 2nd-line treatment (**Figure 1**). No significant differences concerning the absolute counts of the main immune cell populations were identified by groups comparison (**Table 2**). Absolute count of T, B and NK cells are also available for each patient (**Supplementary Table 5**).

Analysis performed on CD4+ T cell subsets showed significantly lower frequency of recent thymic emigrants (RTE) and naïve T cells in AIC-sIEI patients compared to AIC, with an increase of T CD4+ central memory (CM) compartment (**Figures 2A–E**). The same imbalance between naïve and memory compartments was observed for cytotoxic CD8+ T cells (**Figures 2F–I**). As Treg cells play a pivotal role in peripheral homeostasis, we also evaluated their total frequency, as well as the fraction of naïve and memory Tregs. Patients with AIC-sIEI presented a heterogeneous distribution of Treg subpopulations when compared to the AIC-only group. We also observed a reduction of total ($P<0.05$) and naïve Tregs, and an increase of memory Treg compartment, as detected for the other T cell lineages (**Figures 2J–L**).

T cell subsets frequencies were then analyzed by PCA. Notably, among the AIC-sIEI group (red triangles), 11 patients out of 15 defined a specific subgroup, uniformly distributed in an area far from the AIC-only group that skewed towards CD4+ and CD8+ memory T subsets, while other 4 patients lay inside the AIC-only group area (grey dots) (**Figure 3**).

Concerning TCRαβ double negative T cell (DNT) evaluation before treatment, two patients (P14 and P18) were found to be in ALPS-range (i.e., >6% of CD3+TCRαβ+ T cells) (38), while other patients displayed borderline DNTs (**Supplementary Table 1**).

Surprisingly, we did not detect any significant difference within B cell subsets, including CD21 low B cells, by univariate (**Supplementary Figure 1**) and PCA analysis (data not shown). However, we observed very low switched memory B cell frequencies in patients with hypogammaglobulinemia, as previously described (39).

TABLE 1 | Clinical features related to each cytopenia group.

	AIC-alone (N = 9)	AIC-sIEI (N = 21)
General Features		
Gender (M/F)	8/1	13/8
Median age at onset (y; range)	5; 1-15	9; 2-24
Autoimmune Cytopenia Diagnosis (N)		
AIHA	0	8
ITP	5	13
AIN	4	5
Trilineage cytopenia	1	4
Immunological Features (N)		
Family history of immune disorders	1	7
Hypogammaglobulinemia	0	8
Auto/Hyper-inflammation	0	2
Organ-specific autoimmunity	0	5
Splenomegaly	0	10
Lymphadenopathy	0	7
Malignancy	0	1
Recurrent infections	0	5
Major infections	0	1

AIHA, ITP and AIN counts include both mono- and bi-lineage cytopenia cases. N, count; y, years.

TABLE 2 | Absolute counts and frequencies of T, B and NK cell populations.

Population	AIC-alone		AIC-sIEI		*p*-value
	N	Mean (SD)	N	Mean (SD)	
Lymphocytes count (x10^9/L)	7	2,40 (1,48)	15	2,22 (2,98)	0,86
CD3 T cells count (x10^9/L)	7	1,66 (0,94)	15	1,02 (0,51)	0,14
CD4 Helper T cells count (x10^9/L)	7	1,07 (0,64)	15	0,57 (0,31)	0,09
CD8 Cytotoxic T cells count (x10^9/L)	7	0,42 (0,25)	15	0,33 (0,21)	0,42
CD19 B cells count (x10^9/L)	7	0,31 (0,43)	13	0,95 (2,73)	0,42
CD56 NK cells count (x10^9/L)	7	0,11 (0,09)	14	0,20 (0,18)	0,15

Mean, standard deviation (SD) and p-value of AIC-alone and AIC-sIEI group are shown. N, count.

Effects of Immunomodulatory Treatment on T Cell Subsets

Patients presenting with chronic/refractory AIC require a differential clinical management than patients with acute, transient AIC, which may need to be further adapted in presence of additional signs of immune dysregulation (13). In particular, in our cohort a higher proportion of AIC-sIEI patients underwent 2nd- and 3rd-line treatment, and for 6 patients the severity of their clinical status led to the choice of HSCT as definitive therapy. Conversely, none of the patients only presenting with AIC required HSCT, and those cases with isolated neutropenia needed no treatment (N=3) (**Table 3**).

In order to assess the treatment effect on T lymphocyte subsets, we compared immunophenotypic data obtained from AIC-alone and AIC-sIEI groups both before and after treatment with immunomodulatory agents (MMF and/or sirolimus) by PCA (**Figures 1** and **3**). Upon therapy, patients with isolated AIC did not significantly change their position in the PCA plot. On the other hand, AIC-sIEI subjects shifted towards the naïve area of the diagram, with the exception of P13 and P18 who segregated independently, suggesting a different clinical response to treatment (**Figure 3**).

Identification of Variants in IEI-Associated Genes

Based on immunophenotyping results, we performed genetic analysis in patients with family history of immune disorders and/or signs of IEI, in order to identify the molecular bases of the observed immunological defect. Due to the advances made in

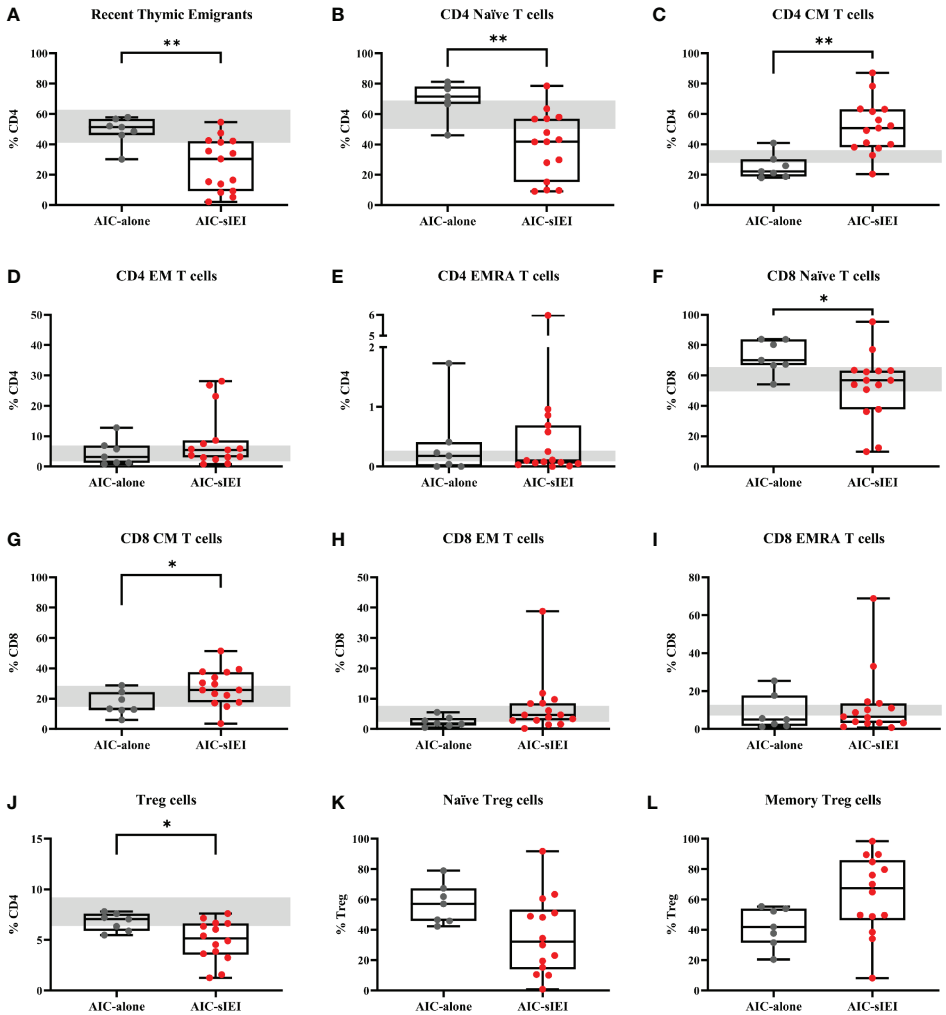

FIGURE 2 | T cell subpopulations immunophenotyping analysis before 2nd- and 3rd-line treatment. Lymphocyte frequencies data (%) of AIC-alone (N=7) and AIC-sIEI (N=15) patients relative to **(A–E)** helper CD4+ T cells, **(F–I)** cytotoxic CD8+ T cells and **(J–L)** Treg subpopulations. **(L)** Treg subsets were not available for P11. Box plots show the 25th percentile (bottom edge), 50th percentile (median) and 75th percentile (top edge); vertical lines at the top and bottom indicate minimum and maximum values. Grey bars indicate control range, based on age-matched median values (35–37). p-values <0.05 (*) or <0.01 (**) are indicated. CM, central memory T cells; EM, effector memory T cells; EMRA, terminally differentiated effector memory T cells.

sequencing technology, more than half of the patients underwent targeted NGS panel sequencing (14/21, **Supplementary Table 4**), comprising 58 genes, while in the remaining ones (7/21) WES analysis was performed (34).

Strikingly, genetic analysis of 12/21 AIC-sIEI subjects was inconclusive or needed further investigation, which is currently underway. Of note, two of these (P20 and P29) had clinical and immunological features of common variable immunodeficiency (CVID). Conversely, 9/21 patients presenting with clinical features of immune dysregulation displayed disease-associated variants in the following genes (**Figure 4** and **Table 4**): *FAS, UNC13D, STAT3, CARD11, PIK3CD, KMT2D, IKZF1*, and *AIRE*.

We identified a T158fs *FAS* mutation in P14, presenting with both a family history and clinical signs of autoimmune lymphoproliferative syndrome (ALPS), including high DNT frequency (21.75%) and AIHA. Coherently, he also displayed a reduced FAS expression in T cell subsets. Since other family members carried the same mutation, despite a less profound impact on protein expression, we hypothesize that P14 may also present a somatic loss of heterozygosity (sLOH) in the DNT population (40). Sanger sequencing of DNA extracted from sorted DNTs is currently ongoing.

Two loss-of-function (LOF) mutations in *UNC13D* (I848L and A995P, in *cis*) were found in P12, who clinically displayed chronic ITP and lymphoproliferation. These findings are in accordance with a previous report that considered the same *UNC13D* variants as predisposing to ALPS development (41). Interestingly, a novel variant in *UNC13D* gene (R1075W) was detected in P22, who presented a CVID-like clinical phenotype

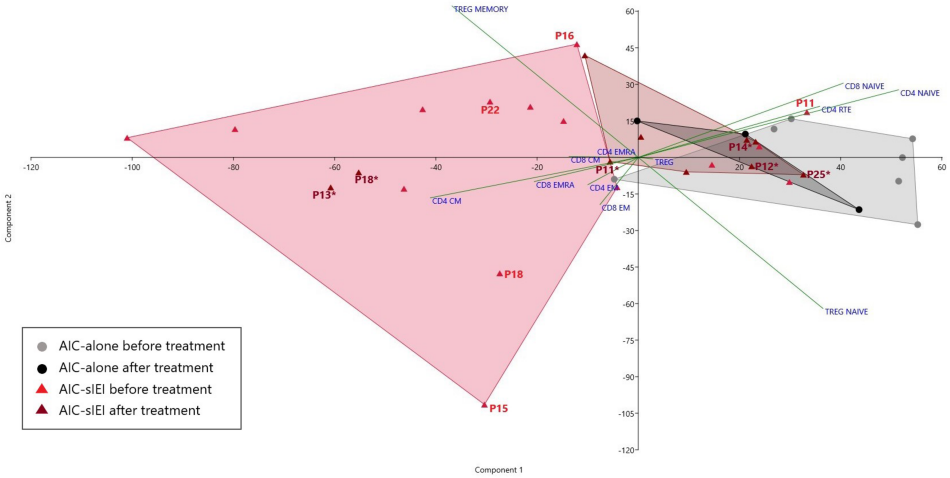

FIGURE 3 | PCA of T cell subsets frequencies before and after 2nd- and/or 3rd-line treatment. Scatter plot displaying the distribution of T cell subsets frequency for AIC-alone and AIC-sIEI patients pre- and post-immunosuppressive (2nd- and/or 3rd-line) treatment (AIC-alone: N=7 before treatment, N=3 after treatment; AIC-sIEI: N=15 before treatment, N=10 after treatment). Grey and red areas indicate patients' clusterization. AIC-sIEI patients who underwent genetic analysis are indicated for both time points (P, before treatment; P*, after treatment).

TABLE 3 | Patients' lines of therapy.

Treatment	AIC-alone (N = 9)	AIC-sIEI (N = 21)
No treatment	3	1
1st-line treatment	6	15
Failed 1st-line treatment	4	9
2nd- or 3rd-line treatment	4	13
HSCT	0	6

AIC first-line therapy included intravenous immunoglobulin (IVIG) and/or corticosteroids; second- and third-line therapy MMF, sirolimus, rituximab, and eltrombopag. HSCT, hematopoietic stem cells transplantation; N, count.

with bilineage autoimmune cytopenia (AIN+ITP) and hypogammaglobulinemia.

A *de novo* heterozygous germline *STAT3* P715L mutation previously described (42–44) was identified in P18, presenting with life-threatening AIHA and other clinical findings associated with STAT3 gain-of-function (GOF) (44, 45).

Molecular investigations performed on P16, presenting with AIHA, family history of autoimmunity, celiac disease and splenomegaly led to identification of the I544L gene variant. The variant was previously reported as benign, although autoimmune features - including cytopenias - have already been associated with hypomorphic *CARD11* mutations (46).

A known E525A *PIK3CD* mutation was detected in P15, who presented with lymphadenopathy, splenomegaly and AIHA. Based on these genetic and clinical findings, Activated PI3Kδ Syndrome (APDS) was diagnosed (47, 48).

Kabuki syndrome (KS), a rare multisystemic immune disorder, was diagnosed in P13 carrying the novel heterozygous E1738* mutation in *KMT2D* gene (49). The patient displayed typical dysmorphic features, chronic ITP and recurrent infections, which have been previously reported in other KS patients (50, 51).

A heterozygous R502L mutation in *IKZF1* gene was identified in P25, who came to our attention for Burkitt lymphoma, and subsequently developed AIN and ITP. Functional studies revealed that this genotype leads to reduced protein stability and to impaired IKAROS homo- and heterodimerization by haploinsufficiency (52).

We found the *AIRE* V301M heterozygous mutation in P11, displaying acute and persistent AIN and ITP. Homozygous *AIRE* mutations cause Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED). Cytopenias have rarely been reported in APECED, even though P11 lacks other typical features and disease-specific autoantibodies (53, 54). However, heterozygous *AIRE* mutations - including V301M - may hide behind common autoimmune disorders, and lead to variable clinical manifestations among family members (55, 56).

DISCUSSION

This study confirms the strong relationship between AICs and IEI (13, 16), focusing on the potential role of extensive multiparametric flow cytometry and PCA as screening tools for an underlying genetic disorder. T cell phenotypes analyzed before 2nd- or 3rd-line treatment revealed an imbalanced T CD4+ and CD8+ profile in patients with AIC-sIEI. In particular, we observed a significant predominance of the mature/memory T cell compartment, counterbalanced by a reduction of T naïve and RTE subsets. Moreover, a reduced Treg frequency was detected in the AIC-sIEI group. These findings suggest the presence of an underlying immune dysregulation that skews the T cell-mediated response towards an activated status. In a clinical context, this corresponds to autoimmune features with or without lymphoproliferation, which are typically associated with PIRDs (57).

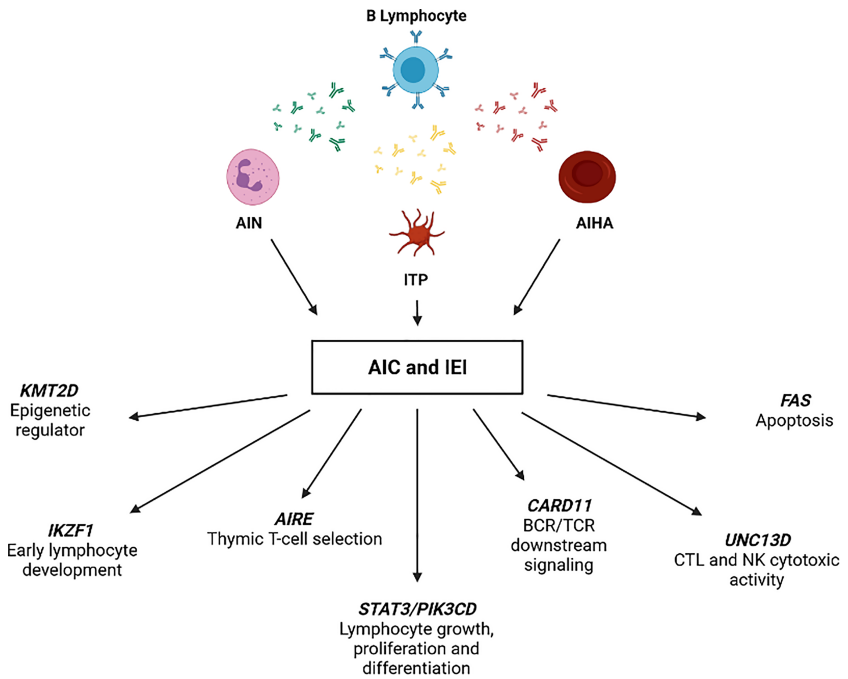

FIGURE 4 | IEI-associated gene variants identified in patients with AIC and signs of immune defect (AIC-sIEI).

The heterogeneity of lymphocyte frequency data is in line with the high variability of IEIs that may clinically display autoimmune cytopenias (8, 13). These include CVID, which typically bears abnormal B cell subsets including a reduction in switched memory B cells (CD19+CD27+IgD-) frequency (58), especially in patients with autoimmune features (59). The scant number of CVID cases in our cohort (P20 and P29 only) may justify the lack of statistical significance in the frequencies of CD19+CD27+IgD- cells between the AIC and AIC-sIEI groups, as well as for other B cell subsets (e.g., CD21low). Interestingly, evidence suggests that the risk of autoimmunity in CVID is particularly increased in patients bearing a reduction in naïve CD4 cells, RTEs, naïve CD8 and

TABLE 4 | Genetic results of patients presenting with AIC associated with sings of PIRD.

Patient	Gene	OMIM and Inheritance	cDNA mutation	Zygosity	Protein mutation	HGMD Accession number	VAF	CADD	Protein function
P11	*AIRE* NM_000383	240300 AD/AR	c.901G>A	Heterozygous	p.V301M	CM003856	<1%	25,4	LOF
P12	*UNC13D* NM_199242	608898 AR	c.2542A>C c.2983G>C	Heterozygous in *cis*	p.I848L p.A995P	CM137111 CM137110	<1% <1%	17,16 14,29	LOF
P13	*KMT2D* NM_003482	147920 AD	c.5212G>T	Heterozygous	p.E1738X	CM146820	<1%	3,5	LOF
P14	*FAS* NM_000043	601859 AD	c.471_474delGACA	Heterozygous	p.T158fs	–	–	–	LOF
P15	*PIK3CD* NM_005026	615513 AD	c.1574A>C	Heterozygous	p.E525A	CM1619250	<1%	26	GOF
P16	*CARD11* NM_032415	616452 AD/AR	c.1630A>C	Heterozygous	p.I544L	CM2021163	<1%	0,27	LOF
P18	*STAT3* NM_139276	615952 AD	c.2144C>T	Heterozygous	p.P715L	CM1713821	–	24,9	GOF
P22	*UNC13D* NM_199242	608898 AR	c.3223C>T	Heterozygous	p.R1075W	VUS	<1%	9,13	LOF
P25	*IKZF1* NM_006060	616873 AD	c.1505G>T	Heterozygous	p.R502L	CM212882	–	34	LOF

Details on mutation, frequency (VAF, Variant Allele Frequency), CADD (Combined Annotation Dependent Depletion) score and impact on protein function are shown. CADD score integrates different genomic features such as surrounding sequence context, gene model annotations, evolutionary constraint, epigenetic measurements and functional predictions (33). VUS, Variant of Unknown Significance.

Treg counts (60–62). These findings are surprisingly superimposable to our immunophenotyping results in the AIC-sIEI group, implying that such imbalanced T cell profile clinically correlates with autoimmunity not only in CVID but also in the entire PIRDs galaxy.

Moreover, immunophenotyping revealed elevated levels of DNT cells (38) in two patients: one affected by ALPS-FAS (P14) and the other bearing a *STAT3* GOF mutation (P18), which has recently been depicted as a possible cause of ALPS-Undetermined (ALPS-U) (63). Other patients in both groups displayed borderline DNTs, consistent with recent findings in other autoimmune contexts (64, 65). Indeed, our immunophenotypic results actually agree with an approach based on clinical and family history to select patients that should undergo molecular testing.

PCA performed on pre-treatment T cell immunophenotype showed that patients belonging to the AIC-sIEI group uniformly cluster in an area skewed towards the memory compartment (**Figure 3**), consistent with the presence of an underlying immune dysregulation. Such finding confirms the relevant role of PCA in classifying IEIs (28), and paves the way for its potential usefulness as a screening tool for patients with AICs deserving further genetic analyses. Interestingly, post-treatment PCA revealed a counter-shift of AIC-sIEI patients towards an equilibrium of naïve and memory T cell frequencies. On the other hand, treatment did not significantly impact on the position of the AIC-alone cluster in the PCA plot. This phenomenon highlights that immunomodulatory therapy (MMF and/or sirolimus) determines a partial rebalance of immune dysregulation in the AIC-sIEI subjects, consistent with a good clinical response. Therefore, these drugs might be an early treatment choice for patients with chronic/refractory AICs associated with signs of IEI, and their use should be considered according to the patient's clinical status, as previously proposed (20). Further studies will be required to define the best therapeutic strategy for AIC patients carrying a still undiagnosed IEI.

Of note, two patients remained in the T memory area of the PCA plot after therapy: P13 and P18, respectively affected by KS and STAT3 GOF disease (49, 66). For P18, such lack of response was most likely due to the life-threatening clinical contingency that brought the patient directly to HSCT (44), without attempting targeted treatment with JAK-inhibitors and tocilizumab (26). The complex immunologic background of KS, due to an altered methylation of crucial transcription factors (51), may explain the persistence of T memory-skewed subsets in P13, which could possibly be reversed only by future applications of epigenome editing (67). Therefore, we may speculate that unbalanced immunophenotypes after immunomodulant therapy can act as a warning sign for the need, in highly selected patients, of additional treatment steps such as HSCT or - if available - targeted drugs. Further studies are needed in order to clarify this aspect.

Importantly, genetic analysis showed that IEI-causing mutations were detected in patients displaying suggestive clinical features or a positive family history (AIC-sIEI group, **Table 1**). This finding confirms previous results of a recent retrospective study (13), highlighting that associated clinical signs together with extended immunophenotyping (16) should guide physicians in the decision of performing genetic testing. Notably, 12/21 AIC-sIEI subjects had an inconclusive genetic analysis and are undergoing additional investigations, as well as P11 (*AIRE*), P16 (*CARD11*) and P22 (*UNC13D*) whose WES is currently being processed to rule out whether other mutations may cause the clinical phenotype. Given the increasing number of genes associated with IEI (1), especially within the PIRD microcosm (4), we cannot exclude that future reinterpretation of WES may unravel novel IEI-causing genotypes.

Overall, we identified several genetic causes of immune dysregulation, whose immunophenotypic behavior before and/or after immunomodulant therapy is potentially explainable. In ALPS (P14), for instance, sirolimus has already demonstrated to induce a partial normalization of biomarkers (22). On the other hand, ALPS-like disorders such as STAT3 GOF disease (P18) and APDS (P15) (68–70), as well as *CARD11* loss-of-function mutations (P16) (46), distort intracellular signaling cascades, leading to the previously described altered immunophenotype. Interestingly, hyperactivation of PI3Kδ (P15) enhances mTOR signaling, skewing the differentiation of CD8+ T cells towards short-living effector cells and impairing the development of memory T and B cells (71). Such mechanism gives a possible explanation to the peripheral position of P15 in the pre-treatment PCA plot (**Figure 3**). PI3Kδ's pathway ultimately leads to the suppression of FOXO1, a transcription factor supporting critical genes for lymphocyte development, including IKAROS (P25, heterozygous *IKZF1* mutation) (48, 72). Nevertheless, P25's immunophenotype after therapy shows adequate frequencies of naïve T cells - similarly to other patients with IKAROS dimerization haploinsufficiency (52). Finally, P11 (heterozygous *AIRE* mutation) displayed elevated T naïve frequencies compared to other AIC-sIEI patients, which normalized upon treatment (**Figure 3**). Interestingly, P11 presented a decrease in RTE frequencies, similar to previous reports in APECED (73, 74): such finding may potentially support the contribution of the V301M *AIRE* variant to the patient's complex autoimmune phenotype.

Interestingly, we observed a lower frequency of AIC-alone patients in our cohort compared to recently published studies (13, 16), which could be ascribable to the different inclusion criteria and to the prospective nature of our work. Moreover, two AIC-sIEI patients presenting with multi-lineage cytopenia (P27 and P28) also displayed autoimmune hepatitis (AIH), which it is known to be associated with severe aplastic anemia (SAA). Nevertheless, an aplastic etiology was ruled out performing bone marrow aspirates and biopsies, which revealed a picture compatible with refractory cytopenia of childhood (RCC). Thus, refractory cytopenia was also recently reported as associated with autoimmune hepatitis (75). In light of their clinical behavior and immunophenotyping features, we initially interpreted these cytopenias as immune-mediated, although the clinical evolution revealed over time RCC. Therefore, we decided to include these patients in our study to raise awareness of possible

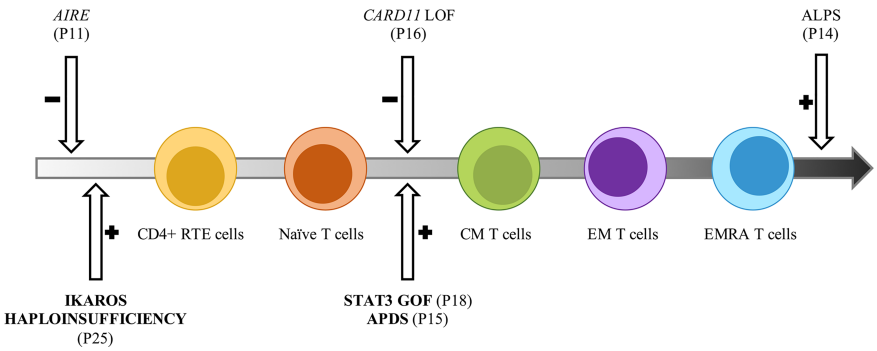

FIGURE 5 | Potential impact of inborn errors of immunity on T cells development and function. Model representative of the T cell subsets alterations observed in the AIC-sIEI group. The gray arrow shows T cell populations skewed towards memory compartment and terminal effectors. Patients harboring a disease-associated (bold) or potentially relevant gene variant are indicated.

overlapping hematological conditions at the time of clinical presentation.

Our real-life study has some limitations, mainly due to the restricted sample size and the scarce number of patients that underwent flow cytometry both before and after treatment. Moreover, immunophenotyping was mainly performed during acute clinical presentation, therefore we cannot exclude that these abnormalities are due to the concomitant inflammatory status, rather than the underlying immune dysregulation. However, a recent retrospective study pointed out similar immunologic alterations in patients affected by AICs with a known genetic etiology (16). Coherent findings in two differently designed studies potentially confirm that the immunologic imbalance detected in our AIC-sIEI population should not be ascribable to the concurrent inflammatory background.

In conclusion, the tight interconnection between hematology and immunology is particularly represented by AICs, which underlie an IEI in a not negligible proportion of cases (13). This study confirms that such relationship is particularly recognizable in PIRDs and further demonstrates the kaleidoscopic presentations of IEI (3), which undoubtedly need a multidisciplinary approach. While clinical signs and family history are paramount to suspect an underlying IEI, extended immunophenotyping and PCA may potentially act as screening tools to identify patients deserving genetic analyses. In our case, patients with a strong suspicion of IEI and those who actually received a molecular diagnosis presented with T lymphocyte subsets significantly skewed towards the memory and effector compartments. Our immunophenotypic results allowed us to build a speculative model explicating how the detected genotypes may impact on specific steps of T lymphocyte's life-cycle (**Figure 5**). Moreover, this study highlights that performing immunophenotyping before and after immunomodulatory therapy may also act as a monitor for treatment response. Larger prospective investigations are needed to improve current knowledge on clinical warning signs of IEI. Achieving a prompt diagnosis may rapidly lead to target therapies (20, 76), or definitive treatments such as HSCT or gene editing (77, 78).

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because according to the protocol approved by Pediatric Ethics Committee for the current study, data sharing is limited to analysis results and not to raw datasets. Requests to access the datasets should be directed to eleonora.gambineri@unifi.it.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

ES, BM, EA, SCM, and MT performed data collection. ES, BM, and SCM performed immunophenotyping analysis. ES, BM, EA, and SCM analyzed data. MLC performed genetic analysis and analyzed data. EC, IF, LL, ID'A, MV, and EG were responsible for patient recruitment and supplied patient care. ES, BM, EA, FC, SCM, and EG wrote the original draft of the article. CF and EG supervised the work. ES and BM have contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Case Report: Hemophagocytic Lymphocytosis in a Patient With Glutaric Aciduria Type IIC

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Hemophagocytic lymphocytosis (HLH) is a rare disease caused by inborn errors of immunity (IEI), secondary to infection, lymphoma or autoimmune disorders, but we often overlook the fact that HLH can be secondary to inborn errors of metabolism (IEM). Here, we describe a patient who was diagnosed with glutaric aciduria type IIC complicated by features suggestive of possible HLH. The diagnosis of glutaric aciduria type IIC, a IEM, was confirmed by whole exome sequencing. The patient was treated with coenzyme Q10 and riboflavin which effectively improved her liver function. During treatment, the patient developed severe anemia and thrombocytopenia. Persistent fever, splenomegaly, cytopenias, increased ferritin, hypertriglyceridemia, hypofibrinogenemia, and hemophagocytosis in the bone marrow pointed to the diagnosis of HLH; however, the patient eventually died of gastrointestinal bleeding. After other potential causes were ruled out, the patient was diagnosed with glutaric aciduria type IIC complicated by features suggestive of possible HLH. When cytopenias occurs in IEM patients, HLH is a possible complication that cannot be ignored. This case suggests a possible relationship between IEM and risk for immune dysregulation.

Keywords: glutaric aciduria, hemophagocytic lymphocytosis, hemophagocytic syndrome, cytopenia, inborn errors of metabolism, IEM

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INTRODUCTION

Hemophagocytic lymphocytosis (HLH) is a rare fatal disease with extremely high mortality rates. It often results from genetic defects in immune system function or due to infections (such as Epstein-Barr virus, Cytomegalovirus, Parvovirus B19), tumors, and autoimmune disorders. HLH may also be caused by inborn errors of metabolism (IEM), a trigger which may often be overlooked (1). Here, we describe an adult with glutaric aciduria type IIC, a IEM, who developed features suggestive of HLH during the diagnosis and treatment of their underlying disease.

Glutaric aciduria is a systemic disease caused by errors in fatty acid oxidation and function of several mitochondrial dehydrogenase enzymes (2). In most cases, this condition has a childhood onset; however, some cases of adulthood onset disease have been reported, possibly due to late-onset

multiple acyl-CoA dehydrogenase deficiency (3). Most patients develop neurological symptoms at the onset of illness (4), accompanied by repeated hypoglycemia (5), hyperlactic acidemia, and hyperlipidemia.

To our knowledge, this is the first case of glutaric aciduria type IIC complicated by HLH. Moreover, this case underscores the importance of considering HLH in patient with IEM and signs as well as symptoms of immune dysregulation. This case also provides evidence for the potential link between IEM and immune dysregulation.

METHOD

Whole Exome Sequencing

The genomic DNA was randomly broken into fragments with a length of 180-280 bp by a Covaris breaker. After end repair and A-tailing, the two ends of the fragment were ligated with adapters to prepare a DNA library. The library with a specific index was pooled with up to 500,000 biotin-labeled probes for liquid phase hybridization, and then the n exons of n genes were captured by magnetic beads with streptomycin, and linearly amplified by PCR. After the increase, the library quality inspection was carried out, and the sequencing could be carried out if it was qualified. After the library was constructed, Qubit 2.0 was used for preliminary quantification, and then Agilent 2100 was used to detect the insert size of the library. After the insert size meet expectations, qPCR was used to accurately quantify the effective concentration (3nM) of the library to ensure the library quality. The library was qualified, and the Illumina platform was used for sequencing according to the effective concentration of the library and the data output requirements. AfterQC was used to evaluate the sequencing quality of the off-machine original sequencing data, and removed low-quality and contaminated reads. The filtered data was sequenced with the human hg19 reference genome using BWA software (Burrows Wheeler Aligner), and then the capture effect was evaluated. GATK software (Genome Analysis Toolkit) was used to analyze SNV (single nucletide variant) and Inde (linsertion and deletion) in the genome. Then the population database 1000 Genomes (1000 human genome dataset), Genome AD (Genome Aggregation Database dataset) 2.1.1 and ExAC (The Exome Aggregation Consortium dataset) was used to filter the analyzed SNV and Indel. The dbNSFP database was used to predict the pathogenicity of missense mutations and splicing mutations. Human Mendelian Inheritance Database (OMIM), Human Gene Mutation Database (HGMD) and Clinvar Database was used to screen for reported mutations. Finally, Sanger sequencing was used to verify all possible pathogenic sites.

Literature Search

A literature search was conducted on PubMed, using the keywords "glutaric aciduria" for case reports and case series written before December 2021 to assess whether this is the first case report of glutaric aciduria type IIC complicated by HLH. Another literature search was conducted on PubMed, using (Inborn errors of metabolism) AND (Hemophagocytic) for case reports and case series written before December 2021 to summarize the cases of IEM complicated by HLH. It should be noted that we did not conduct meta-analysis and systematic reviews, but only reviewed the literature that was queried.

Case Report

A 27-year-old woman had persistent weakness in her upper and lower limbs for 10 years. The weakness of her upper and lower limbs did not affect her work and life. She was misdiagnosed with seronegative polymyositis for which she received 2.5 milligrams prednisone per day one year prior to admission. In the month prior to admission, she gradually became unable to take care of herself. She had no other previous medical history, nor had she traveled abroad. She was not pregnant. The patient's parents were healthy as was her younger brother and son. There was no genetic disease in her family. Her physical examination was normal except for weakness of the upper and lower limbs.

Following admission, she developed repeated episodes of hypoglycemia, hyper lactic acidemia, and hyperlipidemia (**Table 1**). On the fifth day, she was transferred to critical care unit due to respiratory failure, anuria, and liver failure. Both lungs showed large patchy lesions, and the density of the liver was quantitatively measured by CT image as -40 Hu, which was lower than the density of water (0 Hu) and was similar to the density of fat (-40 Hu) (**Figure 1A**). Since the patient showed persistent fever, next-generation sequencing for infectious pathogens and culture of bronchoalveolar lavage fluid were performed to rule out infectious pathogens such as Cytomegalovirus, Herpes simplex virus, Epstein-Barr virus and Pneumocystis in the respiratory tract. The method of mNGS was the same as discribed before (6).

As the patient had hypoglycemia, diagnosis of glycogen storage disease was considered. Due to the patient's persistently abnormal coagulation parameters (**Table 1**), a liver biopsy was not performed. A muscle biopsy did not demonstrate obvious lipid deposits, but magnetic resonance imaging (MRI) of the lower limbs revealed a large amount of fat accumulation between the muscles. The patient remained aurinc due to renal failure, so the urine organic acids were not performed. Results of whole exome sequencing revealed a homozygous mutation of *ETFDH* gene (c.250G>A) as shown in **Figure 1B**. The patient was diagnosed with glutaric aciduria type IIC and was treated with 150mg riboflavin per day and 40mg coenzyme Q10 per day. The patient was alos given a high-sugar and low-fat diet. CT imaging suggested that the patient's liver was improved significantly which was confirmed by laboratory tests (**Figure 3**). Despite this improvement, the patient developed severe cytopenias (45g/L of hemoglobin and 9×10^9/L of platelets).

Severe cytopenias are not typically seen in patients with glutaric aciduria type IIC. Other complicating diagnoses which could explain the findings of cytopenias in glutaric aciduria type IIC were considered. The patient developed acute renal failure with anuria shortly after admission suggesting consideration of thrombotic microangiopathy. Peripheral blood smear findings of

TABLE 1 | Laboratory Data.

Variable	Reference Range	Admission	In ICU HD5	HD15	HD31	Before Death
Hematocrit (%)	38-50.8	43.8	44.2	24.5	12.7	21.7
Hemoglobin (g/dl)	13.1-17.2	14.8	15.2	8.0	4.5	6.3
Platelet count (10^9/L)	83-303	366	314	198	9	270
Red-cell count (10^12/L)	4.09-5.74	4.83	4.94	2.68	1.49	2.3
Mean corpuscular volume (fl)	83.9-99.1	90.7	88.9	91.2	91.4	94.3
Fibrinogen (g/L)	2.0-4.0	1.29	1.23	2.56	2.31	1.33
Activated partial-thromboplastin time (sec)	23.9-33.5	30.4	>150	33.9	34.2	40.7
Alanine aminotransferase (U/L)	9-50	114	179	357	61	20
Aspartate aminotransferase (U/L)	15-40	473	700	636	152	61
Lactate dehydrogenase (U/L)	120-250	ND	2596	1819	ND	ND
Total Cholesterol (mmol/L)	3.14-5.86	10.71	ND	6.83	ND	ND
Triglycerides (mmol/L)	0.3-1.7	8.43	ND	4.28	ND	ND
Lactic acid (mmol/L)	0.5-2.2	3.7	4.3	0.9	1	3.5
Ammonia (μmol/L)	10-47	70	165	98	36	ND
fasting blood-glucose (mmol/L)	3.9-6.1	1.42	4.3	0.5	6.1	6.3
SOFA score			21			9
Apache II score			26			13

HD, hospitalization day; ND, Not done.

thrombotic microangiopathy including mechanical haemolytic anaemia were not found, and pathogenic mutations in genes such as *CD46, CFI, CFB, C3, THBD* and *CFH* were absent (7). Acute fatty liver of pregnancy (AFLP) or hemolysis, elevated liver enzymes, and low platelets (HELLP) were also considered which may occur in pregnant women (8), and in pregnant women with IEM (8, 9); however, the possibility of pregnancy was excluded. NGS and cultures were also performed on samples of

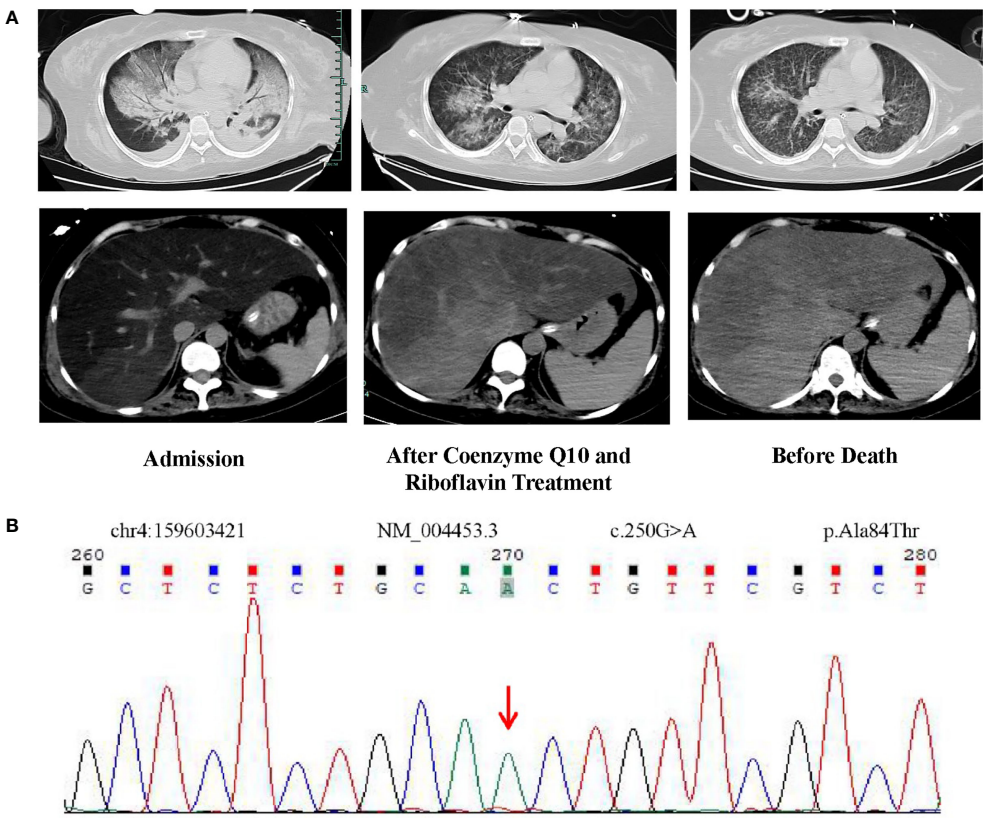

FIGURE 1 | **(A)** CT images of the lungs and abdomen on admission, after coenzyme Q10 and riboflavin treatment, and before death. After treatment with riboflavin and coenzyme Q10, the CT value of the patient's liver gradually increased from -40 Hu at the beginning to nearly normal. **(B)** Homozygous mutation of *ETFDH* gene (c.250G>A) identified by whole exome sequencing.

bronchoalveolar lavage fluid, peripheral blood, and peritoneal fluid, however, no pathogen was isolated which suggested the presence of aseptic inflammation. The persistent and severe hyperlipidemia suggested oxidative stress induced hemolysis; however, peripheral blood smear findings of oxidative hemolysis such as G6PD deficiency were absent. Similarly, whole exome sequencing did not document any pathogenic of G6PD enzyme deficiency-related gene mutations.

Finally, hemophagocytosis was observed on a bone marrow biopsy and aspiration. There are eight diagnostic criteria for hemophagocytic lymphocytosis (1), and the patient met six of them, including persistent fever, splenomegaly (**Figure 1A**), cytopenias (**Figure 3**), increased ferritin (1000 ng/mL, reference range 7-323 ng/mL), hypertriglyceridemia and hypofibrinogenemia (**Table 1** and **Figure 3**), and hemophagocytosis in the bone marrow (**Figure 2**). Of note, serum soluble IL-2R and NK cell activity were not tested in this case. Combined with these laboratory tests, the diagnosis of HLH was suggested. Results from whole exome sequencing showed no gene mutation such as PRF1, UNC13D, STXBP2, STX1, RAB27A, LYST, AP3B1, SH2D1A or XIAP which implied that there was no primary HLH. Besides, no evidence of malignancy, infections, or autoimmune disorders were found. Therefore, we attributed the cause of HLH features to glutaric aciduria type IIC. Considering that this patient had a clear trigger, other treatments (e.g. etoposide, steroids, cyclosporine) were not administered. Supportive care including infusion of red blood cells was performed. Her hemoglobin was maintained at 60g/L, and her platelets gradually increased from 9×10^9/L to normal after the day 32 of hospitalization as liver function continued to improve (**Figure 3**). Unfortunately, she eventually died of gastrointestinal bleeding despite remission of the features of HLH after being hospitalized for a month and a half. The patient's family declined an autopsy.

DISCUSSION

Abnormal blood biochemical examinations such as lactic acid, blood glucose and blood lipids in adults can often lead clinicians to consider the diagnosis of a IEM. In addition to biochemical examinations, whole exome sequencing has aided in the rapid diagnosis of IEM. In this case, the patient showed no obvious neurological symptoms except for upper and lower extremities weakness. The patient's condition progressed to severe hypoglycemia and hyperlipidemia, which is consistent with the clinical manifestations of glutaric aciduria type II. In the east of China, homozygous mutation of *ETFDH* gene (c.250G>A) is the most common cause of glutaric aciduria type IIC (10, 11). This genetic mutation was found in this case (**Figure 1B**). Given that the CT appearance of liver (**Figure 1A**), lipid deposition was suspected. The patient was eventually diagnosed with glutaric aciduria type IIC. Consequently, the patient was administered a high-dose coenzyme Q10 and riboflavin—the two drugs recommended for the disease (2) and the clinical manifestations improved rapidly.

Few people would consider HLH in the differential diagnosis of cytopenias in IEM patients. She had very serious liver damage and hypertriglyceridemia. She also had multi organ failure, including anuria, respiratory failure and liver failure, which made it easy to overlook the HLH features. As a critically ill patient, all of her clinical symptoms were non-specific. After considering and excluding important diagnoses associated with acute onset of cytopenias, the diagnosis of HLH was considered. HLH is a fatal disease which is often caused by genetic defects, or it may develop secondary to malignancy, autoimmune diseases, and infections (1). However, none of these factors were found during the disease course of our patient. Therefore, we attributed the occurrence of HLH to glutaric aciduria type IIC.

In the past 30 years, cases of IEM complicated by HLH have been reported (**Table 2**). Almost all cases occured in children, so in

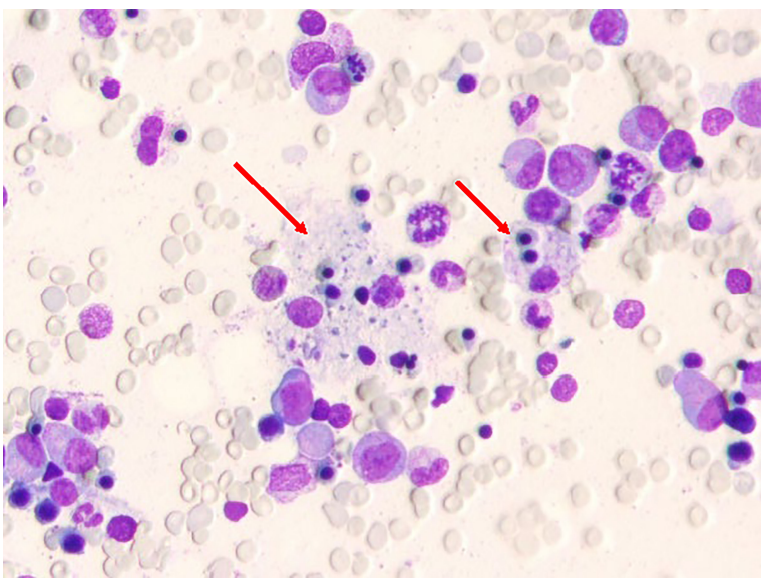

FIGURE 2 | Bone marrow smear carried out when hemoglobin was lowest; the arrow indicates hemophagocytic cells.

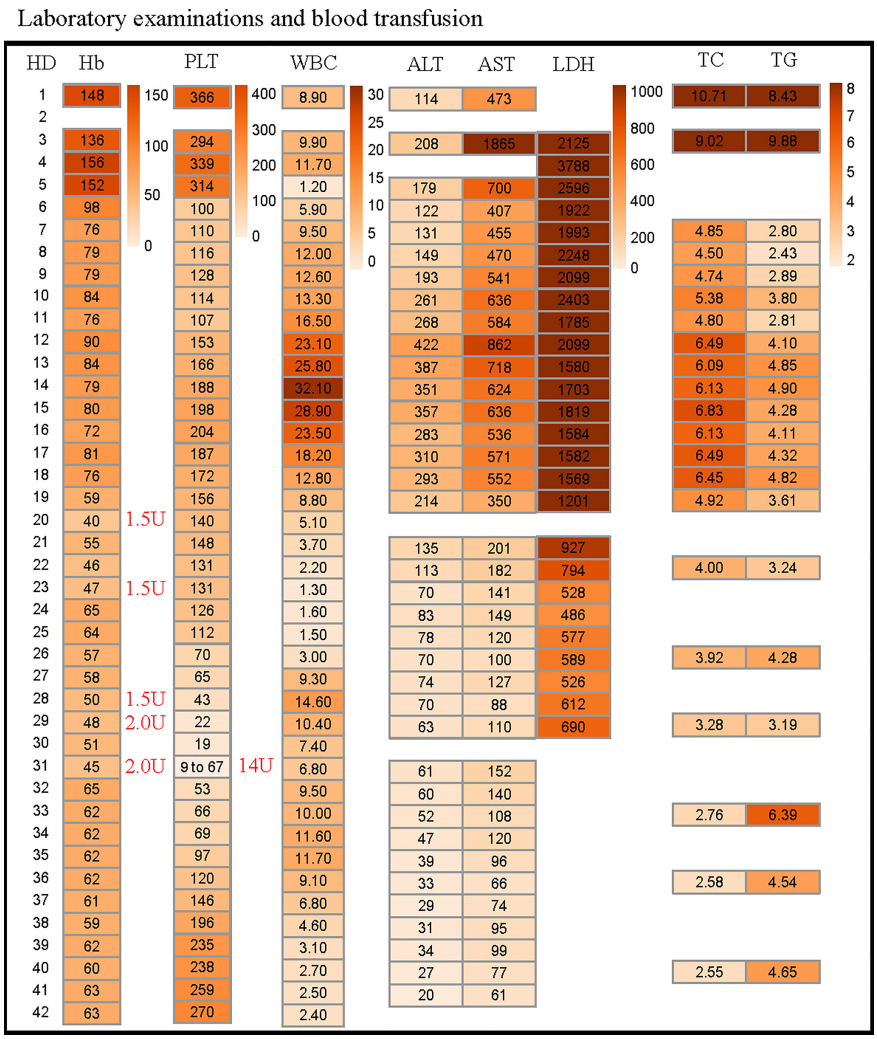

FIGURE 3 | Changes in patient's blood routine, liver function within 42 days after admission. The reference range for each variable are shown in **Table 1**. The amount of red blood cells and platelet administered to the patient are marked with red font on the right side of the corresponding time. HD, hospitalization day; Hb, hemoglobin; PLT, platelets; WBC, white blood cell; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triglycerides.

the treatment of adult IEM patients, the diagnosis of HLH may be overlooked. Some reported cases were associated with Lysosomal Storage Disease (LSD), including Gaucher Disease (GD) (21, 22), Chediak-Higashi Syndrome (CHS) (29), Griscelli's Disease (28), Hermansky-Pudlak Syndrome Type II (HPSII) (23, 24), Wolman's Disease (a type of lysosomal acid lipase deficiency) (25–27). NK cell dysfunction could be found in some LSDs (e.g. CHS, Griscelli's Disease, HPSII) because of lysosomal dysfunction, so it is also classified as IEI. Excluding LSD, many forms of IEM can lead to the occurrence of HLH. Disorders of lipid metabolism such as glutaric aciduria type IIC and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (13) or disorders of organic acid metabolism such as lysinuric protein intolerance (LPI) (19, 20, 35), methylmalonic acidemia (17), propionic acidemia (17, 18) may be complicated by HLH. There are many other rare IEMs complicated by HLH that are also reported such as biotinidase deficiency (12), hepatolenticular degeneration (33), mevalonate kinase deficiency (30, 31), pyrimidine deficiency (32), disorder of glycogen metabolism (14, 15), prolidase deficiency (16) and cobalamin C disease (34).

These signs and symptoms of HLH occurring in the context of IEM indicate that different IEM complicated by HLH have heterogeneity. In addition to the typical symptoms of HLH, most IEM patients with HLH also have many clinical manifestations that may be related to the primary disease. Some patients may develop metabolic encephalopathy (17, 18, 30), and some patients may have severe gastrointestinal symptoms (20, 26, 27). Metabolic acidosis is also a relatively common clinical manifestation in IEM complicated by HLH (17, 32). Treatments may be variable and may include IVIG, etoposide, cyclosporine, plasma exchange and hematopoietic cell transplantation (table 2). Non-LSD IEM patients may not need targeted treatment of HLH features and signs of HLH may regress

TABLE 2 | Reported cases of IEM complicated by HLH.

Reference	Type of IEM	Age at onset of HLH features	Concomitant symptoms in addition to HLH features	Treatment for HLH	Treatment responses	Prognosis
Disorder of energy metabolism						
This paper	*Glutaric Aciduria Type IIC*	27-year-old	hypoglycemia and metabolic acidosis	no treatment	remission	died
Kardas et al. (12)	*Biotinidase Deficiency*	4-month-old	/	IVIG	remission	alive
Erdol et al. (13)	*Long-chain 3-hydroxyacyl-CoA Dehydrogenase Deficiency*	4-month-old	/	IVIG; PE	lack of remission	died
Düzenli et al. (14)	*Type Ia Glycogen Storage Disease*	5-month-old	hypoglycemia	HLH-2004 protocol	remission	alive
Wei et al. (15)	*Type IV Glycogen Storage Disease*	11-month-old	/	dexamethasone; ruxolitinib	remission	/
Disorder of organic acid metabolism						
Rossignol et al. (16)	*Prolidase Deficiency*	all child	/	IVIG, corticoids, and ganciclovir for one confirmed case; cyclosporine and dexametha for one suspected case	patient 1 was remission; patient 2 was lack of remission	/
Gokce et al. (17)	*Methylmalonic Acidemia*	4-year-old	metabolic acidosis and deterioration of consciousness	HLH-2004 protocol; PE	lack of remission	died
Gokce et al. (17)	*Propionic Acidemia*	patient 1 was 2-year-old; patient 2 was 7-year-old	Both patients showed metabolic acidosis and deterioration of consciousness	patient 1 received HLH-2004 protocol and PE; patient 2 received IVIG and cyclosporine	all remission	alive
Aydin et al. (18)	*Propionic Acidemia*	2-month-old	deterioration of consciousness	IVIG and HLH-2004 protocol	remission	alive
Duval et al. (19)	*Lysinuric Protein Intolerance*	all child	/	/	/	/
Ouederni et al. (20)	*Lysinuric Protein Intolerance*	9-month-old	gastrointestinal symptoms	no treatment	remission	alive
Lysosomal storage disease (LSD)						
Sharpe et al. (21)	*Gaucher Disease*	newborn	/	HLH-2004 protocol; HSCT	lack of remission	died
Schüller et al. (22)	*Gaucher Disease*	newborn	/	/	/	/
Enders et al. (23)	*Hermansky-Pudlak Syndrome Type II*	2-year-old	severe bleeding episode	/	/	died
Dell'Acqua et al. (24)	*Hermansky-Pudlak Syndrome Type II*	17-year-old	/	dexamethasone; etoposide	lack of remission	died
Essa et al. (25)	*Wolman's Disease (a type of lysosomal acid lipase deficiency)*	from 2-month-old to 4-month-old	Both patients showed severe gastrointestinal symptoms	/	/	all died
Taurisano et al. (26)	*Wolman's Disease*	4-year-old	severe gastrointestinal symptoms	/	/	died
Rabah et al. (27)	*Wolman's Disease*	2-month-old	severe gastrointestinal symptoms	HLH-2004 protocol	lack of remission	died
Goldberg et al. (28)	*Griscelli's Disease*	all juvenile	/	/	/	/
Rubin et al. (29)	*Chediak-Higashi Syndrome*	11-month-old	/	methylprednisolone; HSCT	lack of remission	died
Other IEM						
Rigante et al. (30)	*Mevalonate Kinase Deficiency*	7-year-old	arthralgias and deterioration of consciousness	methylprednisolone and cyclosporine	remission	alive
Tanaka et al. (31)	*Mevalonate Kinase Deficiency*	all child	/	one patient received the HLH-94 protocol and HSCT; the other one received repeated PE	/	patient 1 died ; patient 2 alive
Pérez-Torras et al. (32)	*Pyrimidine Deficiency*	2-month-old	metabolic acidosis	/	lack of remission	died
Yokoyama er al (33).	*Hepatolenticular Degeneration*	10-year-old	/	methylprednisolone; cyclosporine A; PE	remission after liver transplantation	alive
Wu et al. (34)	*Cobalamin C Disease*	4-month-old	increased creatinine	no treatment	remission	alive

IVIG, Intravenous immunoglobulin; HSCT, hematopoietic stem cell transplantation; PE, plasma exchange; /, not mentioned.

as the primary disease improves (19, 20, 34, 35). For the treatment of IEM complicated by HLH, we recommend that the patient's HLH features be carefully monitored with respect to the response of the treatment of the underlying IEM. In this case, although the patient eventually died of gastrointestinal bleeding, the patient responded well to riboflavin and coenzyme Q10, and her HLH features showed signs of remission which suggests that in patients with IEM, the treatment of the primary disease may be crucial. However, it should be noted that the treatment of such patients still requires the cooperation of metabolic physicians, immunologists, hematologists and intensive care physicians to develop an individualized treatment plan.

However, we cannot clarify the causal relationship between glutaric aciduria type IIC and HLH and we have not explored the pathogenesis which are the limitations of this case report. The rare incidence of IEM and the rare complication of HLH limit the ability to often consider the diagnosis of HLH when faced with a patient with a IEM. Also, due to insufficient knowledge of the potential association of IEM and HLH, many patients may be misdiagnosed. Many potential links between metabolism and immunity have been discovered (36, 37). This case provides evidence for the relationship between IEM and impaired immune function. When cytopenias occur in IEM patients, HLH is a possible complication that cannot be ignored.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of the First Affiliated Hospital of Zhejiang University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

XF, LH, WW, YZ, HY wrote the first draft made the initial diagnosis. XF, LH, LT participate in the discussion of the diagnosis. We all and cared for the patient and reviewed the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.810677/full#supplementary-material

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Primary Immunodeficiencies and Hematologic Malignancies: A Diagnostic Approach

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INTRODUCTION

Primary immunodeficiencies (PIDs) are a heterogeneous group of inherited disorders characterized by aberrant immune function that leads to increased susceptibility to infections and/or immune dysregulation. In addition, some PIDs have an increased predisposition to malignancy. Amongst patients enrolled in the United States Immune Deficiency Network `(USIDNET) registry between 2003 and 2015, there was a 1.42-fold excess relative risk of malignancy compared to the age-adjusted population in the Surveillance, Epidemiology and End Results Program (SEER) database (1). The majority of malignancies were hematological, mainly lymphoid, and linked to the cell type affected by the PID. Significant increases in lymphoma in both males (10-fold) and females (8.34-fold) were observed. B-cell lymphomas are more common and there is an 8-fold increased risk for Non-Hodgkin's Lymphoma (NHL) for all PIDs. Amongst DNA repair disorders, T- cell NHL is more common in ataxia telangiectasia (AT) whereas B-cell NHL is more common in Nijmegen Breakage Syndrome (NBS); malignancy is also the strongest negative factor affecting survival.

PIDs are categorized based on the segment of the immune system primarily involved, as shown in **Table 1**. Lymphoid malignancies are more common in all the categories except for congenital defects of stem cells or phagocytes, which are associated with increased risk of myeloid malignancies and myelodysplastic syndrome (5). We herein describe two cases of PIDs that manifested with a hematologic malignancy and discuss a diagnostic approach including when to suspect an underlying PID and diagnostic evaluation.

Case #1

An 18-month-old boy presented with stridor and cyanosis with crying. Chest x-ray revealed an anterior mediastinal mass. Prior history was remarkable for rotavirus gastroenteritis requiring admission for 5 weeks following his second rotavirus vaccination, and two episodes of otitis media. Growth and development were normal. Parents were first cousins, and family history was notable for a cousin with leukemia at age 2.

TABLE 1 | Primary immune deficiencies and malignancy: pathophysiology, clinical presentation and diagnostic approach (2–4).

PID Category and Examples	Malignancy	Defective Mechanisms	Clinical Features	Investigations
SCID/CID: CARD11 deficiency DOCK8 deficiency ZAP70 deficiency CD40, CD40 ligand deficiencies	Lymphoma, B cell NHL	lymphocyte development co-signaling cytotoxicity tumor immunosurveillance	viral infections fungal infections bacterial infections opportunistic infections autoimmunity	PID diagnostic panel* T cell phenotyping Lymphocyte proliferation Rule out HIV Genetic testing
CID with syndromic features: cartilage hair hypoplasia Wiskott-Aldrich syndrome STAT3-HIES DiGeorge Syndrome	Lymphoma, *May be EBV+ with some*	lymphocyte development co-signaling cytotoxicity tumor immunosurveillance	viral, fungal, bacterial infections opportunistic infections autoimmunity	PID diagnostic panel* T cell phenotyping Lymphocyte proliferation Rule out HIV Genetic testing
		Disease specific: WAS: WASp-dependent rearrangements of the cytoskeleton and modulation of transcription and cell proliferation CHH: cell-mediated cytotoxicity and NK-like activity impaired	**Disease specific:** WAS: low level and small size of platelets CHH: short stature, short hair	**Disease specific:** WAS: flow cytometry for WASp Hyper IgE: Th17 flow cytometry DiGeorge: SNP microarray
DNA repair defects/ defects with radiation sensitivity: ataxia telangiectasia Nijmegen breakage syndrome DNA Ligase IV deficiency Artemis	T-cell lymphoma, B-cell lymphoma, Leukemia	genetic instability	variable susceptibility to infections increased toxicity to chemotherapy Disease specific features: AT-ataxia and telangiectasias NBS- characteristic facial features	PID diagnostic panel* Chromosome breakage Radiosensitivity assays Genetic testing Disease specific: AT: alpha-fetoprotein NBS: chromosomal breakage
Humoral defects: Common variable immune deficiency (CVID) Activated p110δ syndrome Hyper IgM syndromes NFKB1 NFKB2	B cell lymphoma	co-signaling malignant transformation	recurrent sinopulmonary infections bronchiectasis lymphoproliferation autoimmunity	PID diagnostic panel* B cell phenotyping Pneumococcal vaccine challenge Genetic testing
Primary immune regulatory disorders: Autoimmune lymphoproliferative syndrome CTLA4 deficiency LRBA deficiency	Lymphoma	apoptosis tissue inflammation tumor surveillance	autoimmunity lymphoproliferation	PID diagnostic panel* Genetic testing Disease specific: ALPS: double negative T cells, ALPS panel CTLA4, LRBA: flow cytometry

(Continued)

TABLE 1 | Continued

PID Category and Examples	Malignancy	Defective Mechanisms	Clinical Features	Investigations
Defects with increased susceptibility to EBV induced lymphoproliferation: Familial hemophagocytic lymphohistiocytosis X-linked lymphoproliferative disorder MAGT1 CD27, CD70, RASGRP1, CTPS1, CORO1A, CVID	Lymphoma, usually B cell and EBV positive	T and NK cell cytotoxicity tumor surveillance co-signalling	HLH lymphoproliferation chronic viral infections: EBV, HSV, HPV, warts autoimmunity	PID diagnostic panel* NK cell phenotyping CD107a/degranulation assays Invariant NKT cells Genetic testing Disease specific: Primary HLH: flow cytometry for perforin, SAP, XIAP expression
Defects of Stem Cells and Phagocytes: GATA2 Severe congenital neutropenia Shwachman Diamond Syndrome	myeloid malignancies, myelodysplastic syndrome	stem cell defects myeloid differentiation	neutropenia cytopenias bone marrow failure	Complete blood count with differential Bone marrow aspirate and biopsy PID diagnostic panel* Genetic testing
			Disease specific GATA2: monocytopenia, other organ features SDS: short stature, pancreatic insufficiency	**Disease specific:** SDS: fecal elastase

**PID diagnostic panel: lymphocyte subsets with T, B and NK cell enumeration, IgG, IgA, IgM, IgE levels, vaccine titers.*

AFP, alpha fetoprotein; ALPS, autoimmune lymphoproliferative syndrome; AT, ataxia telangiectasia; CID, combine immune deficiency; CVID, common variable immune deficiency; DNT, double negative T cells, EBV, Epstein-Barr virus; FTT, failure to thrive; NGS, next generation sequencing; NHL, non Hodgkin's lymphoma; PID, primary immunodeficiency; SCID, severe combined immune deficiency; SDS, Shwachman Diamond syndrome; WAS, Wiskott Aldrich syndrome; WES, whole exome sequencing; WGS, whole genome sequencing.

Biopsy of the mass was consistent with stage III T-cell lymphoma. He was treated with standard protocol intermediate risk chemotherapy. His course was complicated by recurrent infections that included respiratory coronavirus HKU1 and rhinovirus infections, *Pseudomonas aeruginosa* and *candida* wound infections, neutropenic enterocolitis, presumptive lung fungal infection, and disseminated human simplex virus -1 infections. He also developed excess toxicity from chemotherapy: oral mucositis necessitating gastrostomy tube and prolonged neutropenia needing chemotherapy dose reduction. Immunological evaluation following therapy revealed normal T cell subsets, mitogen proliferation, B cell subsets, and immunoglobulins. T-cell repertoire was persistently abnormal and response to pneumococcal vaccination was poor. Alpha-fetoprotein was normal. Cancer predisposition next generation sequencing genetic panel revealed a homozygous c.7179T>G variant of unknown significance in the ATM gene. Chromosome breakage was highly suggestive of AT with 40% of cells with rearrangements involving chromosome 7 and 14. He developed ataxia at 30 months of age. The cousin with leukemia was subsequently found to have the same AT mutation.

Case #2

A 4-year-old male with no past medical history presented with fever and lymphadenopathy. Imaging and biopsy showed stage III Epstein Barr virus (EBV)+ diffuse large B cell lymphoma. Family history was relevant for a maternal uncle that died of idiopathic liver failure in his 20's. He was treated on a standard protocol and achieved full remission but presented again 6 years later with diffuse extranodal disease (stage IV), EBV+, including scalp and skin involvement. He underwent immune evaluation considering his family history, and extensive relapsed disease at a very young age. Evaluation revealed hypogammaglobulinemia with reduced memory B Cells and EBV PCR was 100,000 copies/mL in whole blood. SAP protein expression *via* flow cytometry was absent. Genetic testing through a PID panel revealed a mutation (c.23A>C,p.His8Pro) in SH2D1A confirming the diagnosis of type 1 X-linked lymphoproliferative syndrome (XLP). After achieving remission of his lymphoma, the patient underwent hematopoietic cell transplantation (HCT) as definitive cure.

DIAGNOSTIC APPROACH

When to Suspect PID

Clues in the clinical history and physical examination can aid in the diagnosis of an underlying PID. Early onset or recurrence of lymphoma should raise suspicion for an underlying PID, in particular DNA repair disorders such as AT and NBS. Presentation with a T-cell lymphoma or leukemia as an infant or toddler is a feature of AT (6). Patients with AT have telangiectasias that occur most frequently in the eyes (7). However, these features are usually not evident until 6 years of age (8). Often, ataxia is the first noticeable sign (9). Patients with NBS have distinctive facial features along with microcephaly and growth retardation (10). These features are usually apparent by 3 years of age. Additionally, increased toxicity from conventional chemotherapy as in Case #1 above should raise suspicion for an underlying DNA repair disorder (11).

A history of recurrent sinopulmonary infections or bronchiectasis is suggestive of an underlying humoral (B-cell) deficiency (12). A history of opportunistic infections such as *pneumocystis jiroveci* pneumonia or recurrent/chronic viral infections such as EBV or cytomegalovirus (CMV) infection favors a T-cell deficiency. Presence of severe warts or molluscum contagiosum are also concerning for an underlying T-cell defect such as DOCK8 deficiency. Eczema is also a common manifestation of T-cell disorders, including Wiskott Aldrich Syndrome (WAS), autosomal dominant STAT3 deficient Hyper IgE syndrome (STAT3-HIES) and DOCK8 deficiency (13). Autoimmunity is often a feature of PIDs, particularly primary immune regulatory disorders (PIRDs) (14). History of autoimmune cytopenia such as Evan's syndrome or organ-specific autoimmunity such as type 1 diabetes or inflammatory bowel disease should raise concern for an underlying PID even without a history of recurrent infections (15).

EBV+ B-cell lymphoma can be a manifestation of PID, particularly those that have a high predisposition to EBV driven lymphoproliferative disorder (16). Examples include X-linked disorders such as X-linked lymphoproliferative disorder (XLP) and MAGT1 deficiency, and autosomal recessive disorders such as CD27 deficiency, CD70 deficiency, ITK deficiency, RASGRP1 deficiency, CTPS1 deficiency, CORO1A deficiency and DOCK8 deficiency. Proteins expressed by these genes are essential components of key pathways important for recognition of EBV-infected B cells by T cells and in the activation of the T- and NK-cell cytotoxicity responses toward EBV-infected B cells. Lymphoma is therefore more likely to be of B-cell origin. Common variable immune deficiency (CVID) can also be associated with EBV+ B-cell lymphoma. EBV+ B-cell lymphoma that is widespread at the time of diagnosis, has high histologic grades, and involves extra-nodal tissues, especially the gastrointestinal tract and central nervous system should prompt evaluation for an underlying PID. Other features that favor an underlying PID with a predisposition to EBV+ lymphoma include a history of pulmonary infections, hypogammaglobulinemia, and history of severe viral infections such as varicella, herpes simplex and CMV. Notably, most severe T-cell defects such as severe combined immune deficiency (SCID) tend not to present with EBV+ lymphoma since they develop very early-onset severe infections before they encounter EBV infection. Lastly, a family history of PID or onset of hematological malignancy in children or young adults is also an important clue for an underlying PID.

How to Evaluate

Comprehensive evaluation that includes quantitative and qualitative assessment of both T-cell and B-cell immunity can aid in the diagnosis of an underlying PID as shown in **Figure 1** (17). The results must be compared with age-matched reference intervals as lymphocyte subsets and immunoglobulin levels vary with age. HIV infection must be ruled out in any patient being considered for a PID. Evaluation should include lymphocyte

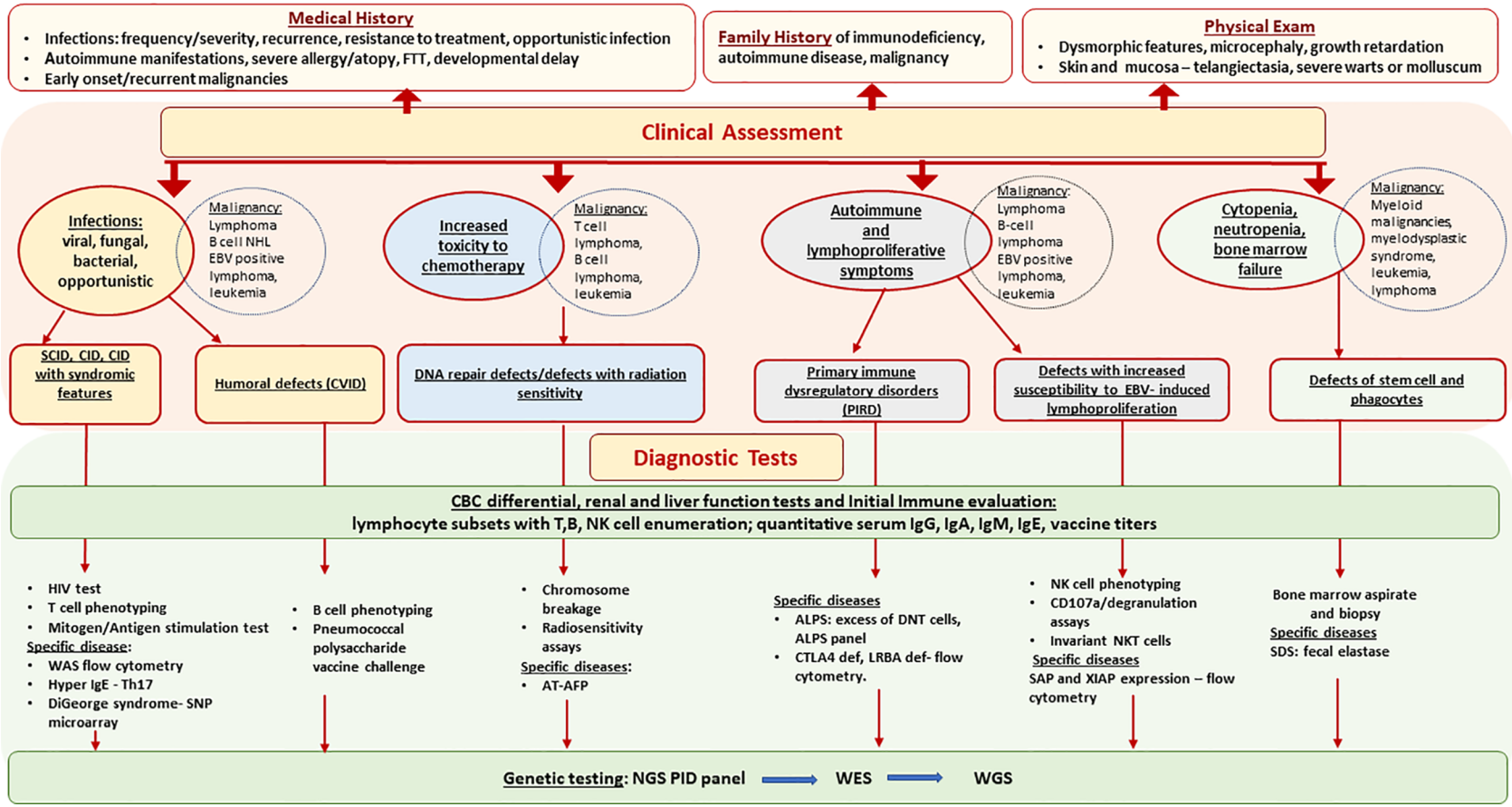

FIGURE 1 | Primary Immunodeficiencies Associated with Hematologic Malignancy - Diagnostic Approach. AFP, alpha fetoprotein; ALPS, autoimmune lymphoproliferative syndrome; AT, ataxia telangiectasia; CID, combine immune deficiency; CVID, common variable immune deficiency; DNT, double negative cells, EBV, Epstein-Barr virus; FTT, failure to thrive; NGS, next generation sequencing; NHL, non Hodgkin's lymphoma; SCID, severe combine immune deficiency; SDS, Schwachman Diamond syndrome; WAS, Wiskott Aldrich syndrome; WES, whole exome sequencing; WGS, whole genome sequencing.

subsets to identify T-cell or B-cell lymphopenia and T-cell phenotyping based on the expression of cell-surface markers such as CD45RA and CD45RO to determine the proportion of naïve and memory CD4 and CD8 T-cells. Low proportion of naïve T-cells (CD45RA+) indicates a defect in T-cell differentiation and thymic output and favors a T-cell deficiency disorder such as AT, NBS, cartilage hair hypoplasia, SCID or combined immune deficiency. T-cell function can be evaluated by assessing proliferative responses to mitogens such as phytohemagglutinin, Concanavalin A or pokeweed and recall specific antigens such as Candida and tetanus toxoid. Impaired T-cell function favors a T-cell deficiency. Radiation sensitivity testing based on flow cytometric-based kinetic analysis of phosphorylated H2AX (γH2AX), ATM, and SMC1 in lymphocyte subsets, should be considered when a DNA repair disorder is suspected (18).

Evaluation of the B-cell arm of the immune system includes measuring the levels of the major immunoglobulin classes IgG, IgA, IgM and IgE. Measurement of specific antibody responses to prior vaccinations such as diphtheria, tetanus and pneumococcal vaccines is useful in identifying defective antibody production. B-cell phenotyping characterizing naïve, transitional, memory and class switched memory B-cells can help identify a defect in B-cell differentiation. Low immunoglobulin levels, non-protective vaccine antibody titers, decreased class switched memory B-cells are suggestive of a B-cell disorder such as CVID or CVID-like disorders such as LRBA deficiency and CTLA4 deficiency. Occasionally, patients with a B-cell disorder can present with a B-cell lymphoma prior to the development of hypogammaglobulinemia but may fail to recover B-cells or B-cell function following rituximab therapy. Hence, it is prudent to perform B-cell phenotyping in patients expected to receive rituximab therapy. In patients with severe allergic phenomena (eczema, eosinophilia, food allergies) measuring IgE levels can also be helpful in identifying certain disorders, i.e., Hyper IgE syndromes such as DOCK8 deficiency, STAT3-HIES and PGM3 deficiency.

Evaluation for autoimmune lymphoproliferative syndrome (ALPS) should be considered if there is a history of autoimmune cytopenia or chronic lymphadenopathy. An ALPS panel is a good initial screening test. Patients with ALPS usually have an increased proportion of T-cells expressing the alpha/beta T-cell receptor but lacking both CD4 and CD8 (α/β-double negative T-cells, aka DNTCs), increase in HLA-DR positive cells (on α/β DNTCs and CD8+ T-cells) with a concomitant loss/absence in CD25+ T-cells and reduced percentage of CD27+ B cells (2). Of note, elevation of DNTCs alone is not pathognomonic for ALPS and can be seen in other immune regulatory disorders.

In patients with EBV+ B-cell lymphoma with features discussed above that raise suspicion for underlying PID, evaluation should include quantification of immunoglobulin levels, B-cell phenotyping and analysis of invariant NK cells. Presence of hypogammaglobulinemia, reduced CD27+ memory B cells, and marked decrease of invariant NKT cells on flow cytometric analysis favors a PID with a unique predisposition to EBV associated lymphoma. Additionally, a history of HLH in boys should raise suspicion for either XLP and MAGT1 deficiency as inheritance is X-linked. SAP expression in T and NK cells should be evaluated by flow cytometric analysis in boys with EBV+ lymphoma or HLH. In patients with a history of HLH, NK-cell studies such as CD107a degranulation and NK-cell function can also aid in the diagnosis of an underlying PID.

Patients with a PID can manifest with a lymphoproliferative disorder (LPD), that can be difficult to distinguish from a lymphoma, either at initial presentation or recurrence. On histopathology, a polymorphic cell population favors a LPD. The presence of a monoclonal process on histology favors malignancy but clonal lymphocyte populations can also be seen in patients without lymphoma (19). Further evaluation with immunohistochemical and gene rearrangement studies are particularly helpful to determine cell lineage as well as to detect genetic/chromosomal aberrations (19).

If the history, physical examination, or immune evaluation are suggestive of an underlying PID, it can be helpful to categorize the type of PID (as shown in **Table 1**.). Genetic testing should be performed as the next step. Obtaining a genetic diagnosis in patients with PIDs is complex because more than 400 different PID-causing genes have been described. Additionally, many patients with similar genetic defects present with variable clinical and laboratory findings. Therefore, unless the evaluation provides an obvious clue to proceed with Sanger sequencing, for example, thrombocytopenia with small platelets and reduced T-cell WAS expression on flow cytometric analysis suggestive of WAS, next-generation sequencing (NGS) involving targeted PID panels is preferred (20). Many PID genes can be evaluated with a single test and current broad based NGS PID panels include >300 genes. Depth of coverage is often excellent and exonic deletions, which commonly occur in several PIDs, are successfully detected (21). If a genetic etiology is not identified despite targeted NGS testing, whole exome sequencing (WES) or whole genome sequencing (WGS) can be considered for second-line genetic testing. With increased accessibility and decreasing costs of testing, WES or WGS can also be considered as a first-line option. It is not uncommon for testing to identify novel variants of uncertain clinical significance (VUCS) in PID genes, making the diagnosis unclear. Further phenotypic and functional characterization can help determine whether the variant is pathogenic or benign.

A diagnosis of PID may guide future therapy and inform prognosis. Discovery of a genetic diagnosis can establish need for allogeneic HCT or identify a potential precision therapy such as leniolisib for activated phosphoinositide 3-kinase δ syndrome (3). In patients with a DNA repair disorder, malignancy is the strongest negative factor affecting survival. These patients would benefit from dose reduction in chemotherapy to limit toxicity. These patients are also at risk of developing future malignancies from imaging procedures that involve increased exposure to radiation such as CT scans and X-rays. Hence, these procedures need to be minimized and done only if absolutely necessary or opt for alternative imaging options such as MRI. Additionally, malignancy can recur in patients with an underlying PID and HCT is often indicated for definitive cure.

In conclusion, clinicians should be vigilant for an underlying PID in patients presenting with a hematologic malignancy. Onset of malignancy at an early age, especially of T-cell origin, a history of recurrent or opportunistic infections, autoimmunity or HLH, dysmorphic features on examination, growth retardation, increased toxicity during chemotherapy, or high grade EBV+ B-cell lymphoma with extranodal involvement should prompt immune evaluation for an underlying PID. NGS genetic testing should be considered early to facilitate the diagnosis of an underlying PID. Identification of an underlying monogenic PID provides important clinical benefits with the potential to alter therapy, impact prognosis and facilitate genetic counselling.

AUTHOR CONTRIBUTIONS

SC wrote the initial draft. TK created and edited the Table and Figure. NAMW and BJD each provided and wrote case summaries and contributed equally to expand the draft. All authors reviewed the complete paper and tables.

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Molecular Diagnosis Is Vital to the Accurate Classification and Management of Thrombotic Thrombocytopenic Purpura in Children

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Thrombotic thrombocytopenic purpura (TTP) is a rare but potentially life-threatening hematologic disease, presenting a myriad of diagnostic and management challenges in children. Here, we provide a review of this disorder and discuss 2 exemplary cases of TTP occurring in adolescents, emphasizing the need for consideration of late-onset congenital TTP (cTTP). We demonstrate the importance of early confirmation of ADAMTS13 enzyme deficiency and the presence or absence of ADAMTS13 inhibitor in order to rapidly initiate the appropriate life-saving therapies. Ultimately, molecular testing is paramount to distinguishing between congenital and acquired immune-mediated TTP.

Keywords: congenital TTP, immune-mediated TTP, pediatric, ADAMTS13, inhibitor

INTRODUCTION

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening hematologic disease, requiring prompt recognition and intervention. It is a rare cause of thrombotic microangiopathy characterized by microangiopathic hemolytic anemia (MAHA), severe thrombocytopenia and high risk for ischemic end organ damage secondary to formation of platelet-rich thrombi in the microvasculature (1, 2). These microthrombi occur in the setting of inadequately cleaved von Willebrand factor (VWF), caused by the absence or severe deficiency of ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) activity. ADAMTS13 is a plasma protein first characterized in 2001, that under normal circumstances cleaves von Willebrand factor (VWF) into smaller multimers (3, 4). Prevention or reversal of end-organ ischemia in TTP is achieved by re-establishing adequate ADAMTS13 enzyme activity, and thereby appropriate VWF cleavage. TTP can be caused by an inherited deficiency of ADAMTST13 activity, resulting in congenital TTP (cTTP). More commonly, however, TTP is due to an acquired deficiency of ADAMTS13 activity resulting from autoantibody-mediated inhibition of plasma ADAMTS13 activity, referred to as immune-mediated TTP (iTTP). Classical forms of TTP may be more readily identified; but atypical presentations of either cTTP or iTTP present significant diagnostic

and management challenges. In this review, we discuss the clinical presentation, diagnostic work up and most up to date therapeutic interventions for TTP, a rare but potentially fatal hematologic disease. We highlight the role of genetic testing to differentiate cTTP and iTTP. To illustrate potential diagnostic and therapeutic challenges, we will refer to two cases, both adolescents, presenting with acute hemolytic anemia and thrombocytopenia, ultimately diagnosed with TTP but requiring very different management strategies.

BACKGROUND

Pathophysiology

TTP is a rare cause of thrombotic microangiopathy defined by clinical criteria, biological markers of intravascular hemolysis, and severe ADAMTS13 deficiency. Severe ADAMTS13 deficiency leads to accumulation and aggregation of platelets *via* ultra-large VWF multimers. Von Willebrand factor, a glycoprotein required for platelet adhesion, is secreted as ultra-large multimers and stored as such until released into circulation upon vascular injury or endothelial cell activation. Under normal physiological circumstances, ADAMTS13 in peripheral circulation facilitates proteolysis of ultra large VWF multimers into smaller forms to prevent abnormal or disorganized activation, with excessive platelet binding. In TTP, however, ADAMTS13 activity is absent or significantly decreased, leading to abnormal platelet adhesion and aggregation due to uncleaved, ultra large VWF multimers, causing formation of diffuse microthrombi within peripheral vasculature (5) (see **Figure 1**). In most cases (>95%), absence of ADAMTS13 activity is due to acquired autoantibodies against ADAMTS13, inhibiting its proteolytic activity. In a minority of patients (<5%), ADAMTS13 deficiency is due to biallelic mutations within the ADAMTS13 gene leading to an inherited chronic deficiency of ADAMTS13, termed congenital TTP (cTTP).

Epidemiology

Global incidence of TTP is reported as 2 to 6 per million individuals with an overall female predominance. Immune mediated TTP (iTTP) represents the vast majority of TTP cases (>95%), generally presenting in late adolescence and early adulthood. Women are more frequently affected than men (~2 to 1). Possible risk factors or triggers that have been linked to iTTP include infections, medications, pregnancy, known autoimmune disease, or evidence of underlying autoimmunity (5). Congenital TTP (cTTP), also referred to as Upshaw-Schulman syndrome (USS), is a very rare autosomal recessive disorder caused by biallelic homozygous or compound heterozygous variants within the ADAMTS13 gene, leading to severely decreased or absent proteolytic activity. More than 200 ADAMTS13 variants across all ADAMTS13 protein domains on chromosome 9 have been identified in patients with cTTP (including missense, non-sense, splice site, and frameshift mutations). Clinical phenotype of cTTP can be quite heterogeneous. Classically, cTTP is thought to present early in childhood; however, more recent case studies have reported patients with milder or even absent clinical signs of disease in childhood but later developing clinically evident hemolysis or small vessel ischemia. Certain pathologic mutations, specifically p.R1060W homozygosity, have been shown to result in residual ADAMTS13 activity, around 5-10% of normal plasma levels, more frequently resulting in this 'late onset' cTTP (6). Like iTTP, active thrombotic microangiopathic events in these patients seem to be triggered by an additional exogenous or endogenous event such as medication use or a state of physiological stress, including pregnancy or infection (7). Importantly however, unlike those with iTTP, all patients with cTTP are at significantly increased risk for transient ischemic attack (TIA) and overt stroke starting at a young age, with up to 20% lifetime incidence of stroke given potential long term ADAMTS13 deficiency (8, 9).

Childhood-onset TTP, defined as initial MAHA episode before age 18, whether cTTP or iTTP, is a rare entity representing only about 5-10% of all TTP cases. A child presenting with an acute episode of TTP presents its own diagnostic and management challenges. However, it is important to note that a higher proportion of pediatric cases represent an initial episode of cTTP highlighting importance for molecular diagnosis. Based on data from the national Registry of the French Reference Center for thrombotic microangiopathies, approximately 35% of newly diagnosed TTP cases in childhood are attributed to cTTP and may present at any age, from neonate to adolescent (10). Pediatric acquired or immune mediated forms of TTP generally present in late teenage years (11). Mortality for untreated TTP, both in pediatric and adult patients, approaches 90%. However, if appropriate therapeutic interventions are initiated promptly at the time of diagnosis, overall mortality is <10%.

DISCUSSION

Clinical Diagnosis

The clinical spectrum of TTP can vary widely; however, the vast majority if not all patients will present with severe thrombocytopenia (platelet count <30 x10^9/L), evidence of microangiopathic hemolytic anemia (including schistocytes on peripheral smear, elevated LDH, indirect hyperbilirubinemia, low haptoglobin, and elevated plasma free hemoglobin) along with potential evidence of end organ damage. Some estimates suggest that up to 60% of patients will have neurological symptoms at presentation, ranging from headache to overt stroke or seizures, and up to 25% may have evidence of cardiac or mesenteric ischemia. Renal manifestations however are typically isolated to proteinuria and/or hematuria, with acute renal failure an unusual finding at the time of diagnosis. Based on reports from the Oklahoma TTP registry, the majority of patients with acute iTTP present with a platelet count of <20 x10^9/L, hematocrit <30% and normal to minimally elevated serum creatinine, a finding particularly helpful when differentiating TTP from other forms of TMA, most notably hemolytic

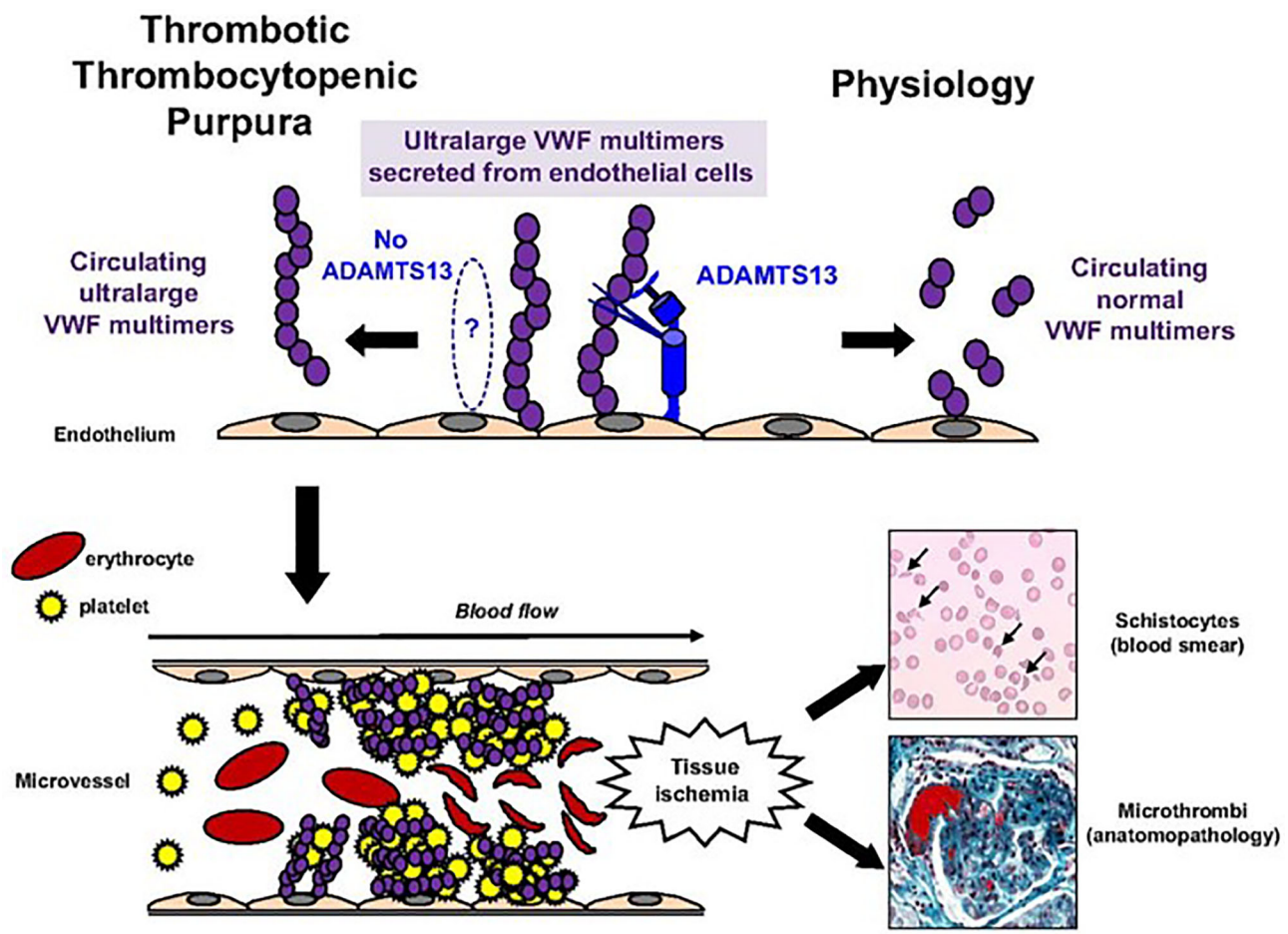

FIGURE 1 | Pathophysiology of TTP. Pathophysiology of TTP. In physiologic conditions, ultralarge VWF multimers released from endothelial cells are cleaved by ADAMTS13 in smaller VWF multimers, less adhesive to platelets. In TTP, because of the absence of functional ADAMTS13 (either absent by congenital defect or inhibited by specific autoantibodies), ultralarge VWF multimers are released into the blood and bind spontaneously to platelets to form aggregates within the arterial and capillary microvessels. The VWF platelet aggregates are large enough to form microthrombi inducing tissue ischemia, platelet consumption, and microangiopathic hemolytic anemia (schistocytes on blood smear).This figure was originally published in *Blood*, and re-printed with permission here. Bérangère S. Joly, Paul Coppo, and Agnès Veyradier. Thrombotic thrombocytopenic purpura. *Blood.* 2017;129(21):2836-2846. © the American Society of Hematology.

uremic syndrome (HUS) (12, 13). Because the signs, symptoms, and laboratory markers overlap with other thrombotic microangiopathies, the distinction of a TTP diagnosis relies on determination of ADAMTS13 activity (2, 14). This is the only biologic marker specific to TTP, and diagnosis is confirmed when ADAMTS13 activity is found to be <10%. However, ADAMTS13 activity levels are often not readily available at the time of presentation in a patient with evidence of MAHA; and in the vast majority of clinical scenarios, the clinician must decide to initiate risky but life-saving therapy before having laboratory confirmation of a TTP diagnosis.

Given this, several clinical scoring systems have been developed to overcome diagnostic challenges and treatment delays related to ADAMTS13 testing turnaround. The goal of these scoring systems – the Bentley score, the French score, and the PLASMIC score – is to identify severe ADAMTS13 deficiency based on the presence of significant clinical predictors alone (prior to confirmation of ADAMTS13 activity) (12, 15–18) (see **Table 1**). Although clinical scoring criteria were developed based on adult cohorts, these criteria may be applicable to pediatric patients presenting with TTP, particularly adolescent cohorts.

We illustrate this diagnostic challenge, in absence of rapid ADAMTS13 testing, given widely variable and often evolving clinical presentation with two adolescent patient cases. The first patient, case 1, is that of a 17-year-old healthy male, with a family history notable for autoimmune disease, who initially presented to an outside hospital following a brief SARS-CoV-2 infection with hematuria, mild normocytic anemia (hemoglobin 11 g/dL), severe thrombocytopenia (platelet count <20 x 10^9/L), and easy bruising. He was diagnosed with presumed immune thrombocytopenic purpura (ITP) and treated with intravenous immunoglobulin (IVIG) x2, with minimal improvement in bleeding symptoms and platelet count. ADAMTS13 testing was

TABLE 1 | Clinical Scoring Systems for the Prediction of Severe ADAMTS13 Deficiency (or TTP).

	Bentley score	French score	PLASMIC score
Predictive Clinical Factors	• indirect hyperbilirubinemia • elevated reticulocyte percentage • thrombocytopenia • normal or minimally elevated creatinine	• creatinine <2.26 mg/dL • platelet count<30 x10^9/L • positive ANA	• platelet count <30x10^9/L • no evidence of malignancy • MCV <90 fL • INR <1.5 • creatinine <2.0 mg/dL • evidence of hemolysis (reticulocyte count >2.5%, undetectable haptoglobin, indirect bilirubin >2 mg/dL)

sent but results were not available at time of discharge, and outpatient hematology follow up was planned.

The second patient, case 2, is a 16-year-old female with past medical history notable for polycystic ovarian syndrome, depression and obesity who initially presented to an outside emergency room with persistent gross hematuria and scattered petechiae. She was found to be severely thrombocytopenic with a platelet count <20 x10^9/L, and a mild normocytic anemia with hemoglobin 9.6 g/dL. She received IVIG therapy for presumed ITP. However, thrombocytopenia persisted, and anemia worsened, with hemoglobin decreasing to 7.6 g/dL over the next 48 hours, along with an increasing reticulocyte count, rising lactate dehydrogenase (LDH), and persistent hemoglobinuria on urinalysis. A bone marrow aspirate and biopsy showed no evidence of malignancy; and with increasing evidence of hemolysis and neurological abnormalities, a presumptive diagnosis of TTP was made. She was then transferred to a large pediatric tertiary care center, with capacity for in-house ADAMTS13 testing, where confirmatory testing was sent.

ADAMTS13 Activity and Inhibitor Testing

ADAMTS13 activity of <10% is necessary to confirm the diagnosis of TTP and is also important in monitoring clinical response to therapy. Accurate measurement of both ADAMTS13 antigen levels (via ELISA) and functional ADAMTS13 activity [via fluorescence resonance energy transfer based assay (FRETS-VWF73) and collagen-binding activity assay] are crucial to the management of TTP. However, just as important to TTP management, is the identification of ADAMTS13 inhibitor. As demonstrated in the cases presented here, this testing is crucial to the distinction between iTTP and cTTP, and ultimately to providing the appropriate management for these patients.

In the case of iTTP, ADAMTS13 auto-antibodies may be either inhibitory or non-inhibitory antibodies, based on their ability to block proteolytic cleavage of VWF *in vitro*. Most patients will have inhibitory antibodies identified, generally IgG; however, 10-15% of iTTP patients have non-inhibitory antibodies that these assays will not detect (5). Traditional assays such as ELISA and FRETS-VWF73 are done *via* incubation of test samples with standard human plasma at varying concentrations, using a standard mixing study. These allow for identification of antibodies leading to ADAMTS13 inhibition *in vitro*. However, two major limitations of these panels exist: false negatives as well as false positives are possible, particularly with collagen-binding based inhibitor panels as they do not allow for identification of non-inhibitory or non-neutralizing antibodies. These are antibodies that do not lead to proteolytic inhibition *in vitro*; however, *in vivo* they lead to severe ADAMTS13 activity deficiency by affecting normal interaction with endothelium or other cellular and plasmatic modulators essential for *in vivo* activity (19–22). These nuances of inhibitor testing must be considered and results interpreted cautiously, with careful correlation to patient's clinical phenotype.

The progression of case 1 illustrates this diagnostic complexity. After his initial presentation, he re-presented to the emergency room one week later with neurological symptoms including memory loss, lethargy, and cognitive slowing. Previously sent ADAMTS13 assay resulted at this time demonstrating undetectable activity (<10%). Inhibitor testing suggested the presence of protease inhibitor (0.9 Bethesda equivalent units, reference range <0.4). Given a high suspicion for iTTP he was immediately initiated on frontline therapy for iTTP (described below). However, he did not exhibit desired response, as he had no significant improvement in hemolytic anemia, thrombocytopenia, or overall functional status. Repeat inhibitor testing as his clinical course progressed (initially utilizing a mixing study, and later enzyme-linked immunoassay [ELISA]) ultimately showed no evidence of ADAMTS13 inhibitor presence. As a result, ADAMTS13 gene sequencing was done demonstrating presence of a homozygous, pathogenic variant *ADAMTS13* c.1584+5G>A (p.)?, which has been previously reported in association with clinical TTP. Parental testing confirmed the cTTP diagnosis, showing each parent to be a clinically unaffected heterozygous carrier of this ADAMTS13 gene variant. This single splice variant, previously reported by Levy et al. in 2001, results in markedly reduced or absent utilization of the normal intron 13 splice donor and activates a cryptic donor splice site at +70, resulting in a 23-codon insertion (3).

Conversely, ADAMTS13 testing for our second patient was notable for an enzyme activity level of 1%, confirming the clinical diagnosis of TTP. She also had a very high inhibitor identified at presentation, based on ELISA testing (88%, reference range <15%), suggestive of proteolytic inhibition likely from IgG autoantibody.

With more than 200 mutations associated with cTTP and clinically relevant limitations of *in-vitro* inhibitor testing as exhibited by case 1, molecular testing should be considered early on, particularly when patients are exhibiting atypical or poor response to immunosuppressive therapy. This is important

to ensure rapid and appropriate therapeutic interventions, as treatment strategies for cTPP and iTTP are distinct.

Treatment

Prompt initiation of medical therapy is crucial in preventing severe morbidity and mortality in TTP. First-line therapy for iTTP is therapeutic plasma exchange: clearing autoantibodies and providing ADAMTS13 in circulation; while ADAMTS13 replacement alone (with FFP, for example) may be utilized for cTTP upfront.

Prior to the advent and use of therapeutic plasma exchange (TPE), mortality rate from acute TTP was near 90%. Its initiation should not be delayed, as immediate TPE has been shown to decrease the risk of mortality related to acute TTP events to <10% (23). Experience and research have shown that in addition to TPE, incorporating adjunct immunosuppressive therapies leads to higher rates of remission and lower rates of relapse; most notably the use of glucocorticoids and rituximab for patients with iTTP (24–28). Upfront rituximab therapy in combination with corticosteroids and TPE therapy has been largely adopted as the front-line standard of care for iTTP (29).

For patients with iTTP, TPE allows for removal of ADAMTS13 autoantibodies, but does not directly target the underlying pathophysiology. Rituximab and steroids allow for immunosuppression and decreased production of antibodies but do not inhibit interaction between existing autoantibodies and ADAMTS13. Caplacizumab, a novel agent, has been shown to provide an important therapeutic role in management of iTTP by targeting a portion of the underlying pathophysiology at play. Caplacizumab is a humanized bivalent variable-domain-only immunoglobulin fragment that targets the A1 domain of VWF (30). It prevents VWF from functioning in platelet recruitment *via* binding of its A1 domain to the GPIb-IX-V platelet receptors, directly targeting the underlying pathophysiology of microthrombosis in TTP. Results of 2 randomized controlled trials have led to approval of caplacizumab for the treatment of adults with iTTP in Europe in 2018 and FDA approval in 2019 (31, 32). International Society on Thrombosis and Haemostasis (ISTH) guidelines support the use of upfront caplacizumab for management of iTTP in adults based on the clinical trials described, despite some potential bleeding risks, still to be defined (33, 34). As expected, our case 2 patient with confirmed iTTP, received TPE and concomitant immunosuppressive therapy. She received 7 days of TPE, with high-dose glucocorticoids (subsequently weaned over ~4 months) and a course of rituximab (375 mg/m^2/dose x 4 weekly doses), an anti-CD20 monoclonal antibody. She demonstrated a rapid response to therapies, with resolution of anemia and thrombocytopenia within 1 week and normalization of ADAMTS13 activity within 2 weeks and resolving hemolysis over the following weeks. She has maintained a durable response to therapy with no evidence of recurrence >6 months from diagnosis.

However, our patient in case 1 was diagnosed with iTTP prior to the results of molecular testing. As a result, immediate TPE and immunosuppression including glucocorticoid therapy, rituximab, and cyclosporine were initiated. He achieved a detectable ADAMTS13 level while on TPE which was short-lived. He did not appear to respond to immunosuppressive therapy and also suffered significant unwanted side effects secondary to high doses of glucocorticoids. Furthermore, irreversible immunosuppressive therapies placed him at prolonged infectious risk prior to his diagnosis of cTTP, which may have been prevented with earlier genetic testing. Ultimately, caplacizumab was initiated. He never achieved detectable ADAMTS13 activity level while receiving caplacizumab therapy; however, interestingly he exhibited significant improvement in hemolytic anemia, thrombocytopenia, and overall functional status. This improvement was short lived, and with cessation of caplacizumab therapy, he again developed clinical signs and symptoms of recurrent TTP.

Alternatively, the mainstay of therapy for cTTP is replacement of the absent or deficient ADAMTS13 enzyme, which has traditionally been done utilizing FFP infusions. However, this can be cumbersome for patients to maintain long-term, requiring frequent administrations within a clinic or hospital setting, and placing patients at risk for antibody development and allergic reactions due to repeated exposures. In addition, an international registry-based review has shown that despite regular prophylaxis with FFP patients remain at relatively high risk for recurrent episodes of TTP, particularly pediatric patients (35). Several studies have explored the use of intermediate-purity factor VIII concentrates for both treatment and prophylaxis in congenital TTP, as certain commercially available products have been shown to contain high amounts of ADAMTS13. Use of such products would potentially allow for less frequent infusions, in-home administration, smaller infusion volumes, and decreased risk of blood-born infections or transfusion-related reactions when compared to standard FFP administration. Several case series report their successful use for this population (36, 37). Studies have analyzed functional and immunoreactive ADAMTS13 in several commercially available products in the US and EU and identified two products (Koate-DVI® and Alphanate®) with high ADAMTS13 activity: 900% and 200%, respectively, compared to activity of 100% in normal pooled plasma, making these products a viable alternative for patients with cTTP (38). Once molecular diagnosis of cTTP was made for our patient in case 1, immunosuppressive therapy was discontinued and a replacement approach was utilized – initially with fresh frozen plasma (FFP), and subsequently with the ADAMTS13-containing factor 8 product Koate-DVI®. He remained clinically asymptomatic without laboratory evidence of recurrent cytopenias or MAHA and was ultimately transitioned to a home prophylactic regimen of Koate-DVI® 50 Units/kg twice weekly, with trough ADAMTS13 activity ranging from 8% to 29%.

More recently, a phase 1 study exploring the safety of the use of BAX930 a recombinant ADAMTS-13 (rADAMTS-13) in patients with severe cTTP demonstrated tolerance and safety, as well as pharmacokinetic profile comparable to that of plasma infusions (39). These results have led to an ongoing phase 3 open label multi-center trial evaluating the safety and efficacy of rADAMTS-13 for prophylaxis as well as treatment for pediatric and adult patients with confirmed severe cTTP (*NCT03393975*).

Conclusion

Because childhood onset TTP is incredibly rare, there are frequent delays in diagnosis of both iTTP and cTTP, which

can lead to inappropriate management or delay in appropriate management, potentially resulting in severe consequences. TTP, particularly when presenting in childhood, may be mistaken for other autoimmune phenomena such as ITP, Evans syndrome, or HUS. As the management of these disorders differs widely, prompt and accurate diagnosis is crucial – first with ADAMTS13 activity measurement and second with ADAMTS13 inhibitor assessment. As demonstrated in Case 1, molecular testing may ultimately be the key to distinguishing cTTP from iTTP, in the setting of uninterpretable inhibitor testing and unclear clinical phenotype, in order to more expeditiously provide the appropriate management for these children and adolescents with TTP.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CK, SK, JE, and AG collected and verified clinical data. CK drafted the manuscript. CK, SK, HH, JE, JD, and AG have all reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Atypical Manifestations Associated With FOXP3 Mutations. The "Fil Rouge" of Treg Between IPEX Features and Other Clinical Entities?

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Introduction: The Forkhead box protein P3 (FOXP3) is a transcription factor central to the function of regulatory T cells (Treg). Mutations in the *FOXP3* gene lead to a systemic disease called immune dysregulation, polyendocrinopathy, and enteropathy, an X-linked syndrome (IPEX) characterized by the triad of early-onset intractable diarrhea, type 1 diabetes, and eczema. An atypical presentation of IPEX has been reported.

Method: We report rare cases with equivocal clinical associations that included inflammatory, kidney, and hematologic involvements screened with massively parallel sequencing techniques.

Results: Two patients with hemizygous mutations of *FOXP3* [c.779T>A (p.L260Q)] and [c.1087A>G (p.I363V)] presented clinical manifestations not included in typical cases of IPEX: one was a 16-year-old male patient with an initial clinical diagnosis of autoimmune lymphoproliferative syndrome (ALPS) and who developed proteinuria and decreased kidney function due to membranous nephropathy, an autoimmune renal condition characterized by glomerular sub-epithelial antibodies. The second patient was a 2-year-old child with bone marrow failure who developed the same glomerular lesions of membranous nephropathy and received a bone marrow transplantation. High levels of

IgG4 in serum, bone marrow, and kidney led to the definition of IgG4-related kidney disease (IgG4 RKD) in this young boy. The circulating Treg levels were normal in the former case and very low in the second.

Conclusion: Two atypical associations of functional mutations of *FOXP3* that include ALPS and IgG4 RKD are described. Membranous nephropathy leading to renal failure completed in both cases the clinical phenotypes that should be included in the clinical panorama of *FOXP3* failure.

Keywords: FOXP3, ALPS, IPEX, membranous glomerulopathy, regulatory T cells, NGS

INTRODUCTION

Immunologic disorders of genetic origin represent a group of diseases characterized by a wide spectrum of phenotypes that frequently pose a diagnostic challenge for possible clinical overlaps (1). They are, in general, monogenic disorders characterized by pleiotropic clinical manifestations, ranging from increased susceptibility to infections to significant immune dysregulation or autoimmunity and hematologic abnormalities, including lymphoproliferation and cytopenia (2). With the advent of next-generation sequencing (NGS), we were able to better classify these disorders based on the underlying mutations.

The immune dysregulation, polyendocrinopathy, and enteropathy X-linked syndrome (IPEX) is an inherited condition associated with the mutation of Forkhead box Protein P3 (FOXP3), a transcriptional factor uniquely expressed by $CD4^+CD25^+$ regulatory T cells (Treg) and closely implicated in the regulation of immune homeostasis (3, 4). IPEX is a life-threatening condition usually emerging in early childhood and characterized by the triad of early-onset intractable diarrhea, type 1 diabetes (T1D), and eczema (5). The spectrum of *FOXP3* mutations may, however, extend beyond the classical IPEX triad, with a still undefined number of cases with an atypical presentation, including late-onset involvement, mild disease phenotypes, and predominant hematologic clinical features (6).

Here we describe two cases with proven pathogenetic variants of *FOXP3* who presented the atypical signs reported in IPEX: a 16-year-old boy who developed an autoimmune lymphoproliferative syndrome (ALPS) with late onset and a 2-year-old child who presented an IgG4-related disease (IgG4 RD) (7) as the first symptom. Kidney involvement occurred at the second stage of the disease in both patients, with similar glomerular lesions of membranous glomerulopathy. The atypical clinical presentation associated with *FOXP3* mutations represents a new syndromic template that should be considered in clinical medicine.

PATIENT 1

The patient is a 16-year-old male with a clinical diagnosis of ALPS (8). At the age of 8 years, he was diagnosed with chronic (> 6 months) lymphoadenopathy. A malignant disorder was excluded, and immunological screening showed high levels of TCR-αβ+-double-negative T-cells (DNTs) and a rise in vitamin B12 serum levels. According to the diagnostic criteria (8), he was diagnosed with ALPS, and the treatment with mycophenolate mofetil (MMF) at 1 g/m^2/day was started with a partial response. At that time, the laboratory tests showed normal renal function and the absence of proteins in the urine.

However, in the following years, the disease symptoms were not completely controlled: at the age of 11, he developed severe thrombocytopenia (PLT 27,000/mm^3) which was refractory to MMF therapy but successfully treated with sirolimus at 2 mg/m^2/day.

At the age of 12, he presented bilateral pan-uveitis treated with local therapy and prednisone, starting with 1 mg/kg/day and tapering until suspension at 1 year later. At the same time, the laboratory tests showed increased creatinine without any changes in the urine tests. The kidney function temporarily improved during steroid therapy but worsened later. Afterwards, he showed complete remission of ALPS-like clinical manifestations. At the age of 16, due to the appearance of proteinuria and persistence of decreased kidney function, the patient was admitted to the Nephrology Unit. A timeline of the events and treatments is presented in **Figure 1**. The serum testing was negative for antinuclear and anti-neutrophil cytoplasmic antibodies, and the serum complement was normal. A renal biopsy was performed. Light microscopy findings included a membranous pattern with 63% (7 out of 11) obsolete glomerulus (**Figures 2A–C**). Active lymphocytic inflammation was present in tubule–interstitium, especially near arterioles. Immunofluorescence microscopy showed glomerular deposition of IgG and C3. The M-type phospholipase A2 receptor (PLA2R) antibody was absent in serum, and the PLA2R antigen was absent in tissue as well as thrombospondin type 1 domain-containing 7A (THSD7A) antigen in tissue (**Figure 2D**). The IgG–IgG4 immunohistochemical staining on kidney biopsy was negative. All investigations are summarized in **Table 1**.

In the light of histological diagnosis, we decided to re-validate the genetic evaluation of the patient that had been tested through an NGS-based gene panel, implementing the human phenotype ontology (HPO) code. We found a mutation at the leucine–zipper domain (exon 8) of the *FOXP3* gene (NM_014009.3): c.779T>A (p.L260Q), never previously reported on GnomAD database (https://gnomad.broadinstitute.org/) and predicted to

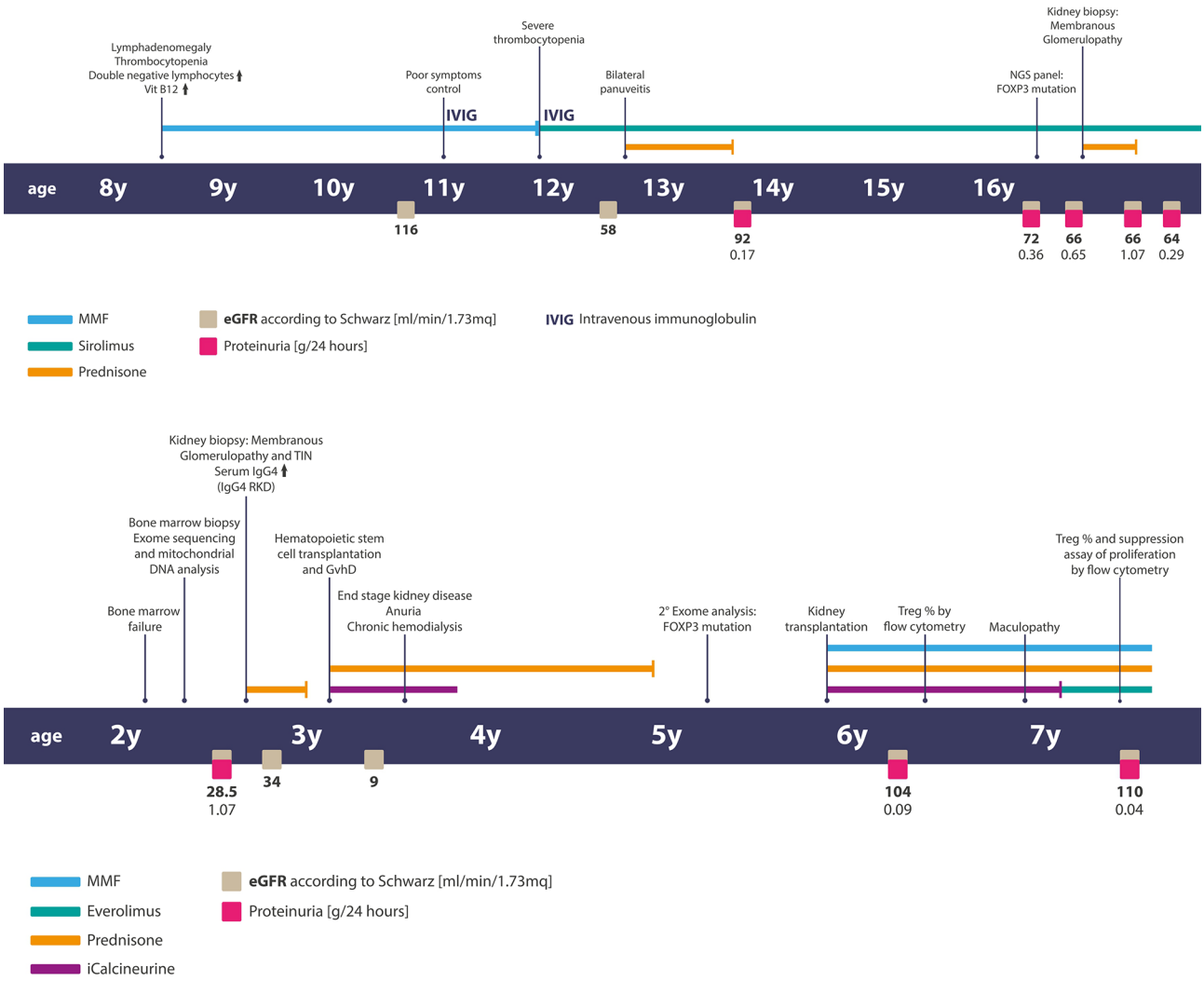

FIGURE 1 | Timelines of clinical events, diagnostic exams, and treatments of patients. Above—the timeline of patient 1 and below— the timeline of patient two.

be likely pathogenic by the Varsome (https://varsome.com/" https://varsome.com/) suite of variant annotation. The proband is hemizygous for the variant inherited from the asymptomatic mother. The patient does not present the classical triad of IPEX (gastrointestinal involvement, cutaneous manifestation, and polyendocrinopathy), and the number of Treg CD4 +CD25+FOXP3+ was in the normal range (2.4%).

He was successfully treated with steroid that started at 1 mg/kg for 1 month with slow tapering and currently continues immunosuppressive therapy with sirolimus, with a good response on proteinuria and with stable kidney function.

PATIENT 2

A previously healthy 2-year-old child was diagnosed with trilinear cytopenia due to bone marrow failure requiring weekly platelet and red blood cell transfusions. The bone marrow biopsy (BMB) showed a severe hypocellularity (20%) with lymphoplasmacytic infiltrate. The screening investigations performed to rule out the diagnosis of Fanconi anemia and telomeropathies were negative (DEB test, telomere length measurement). Congenital bone marrow failure syndromes (cBMFs) were also excluded by a large NGS panel, and the mitochondrial DNA analysis results were normal as well. The IgG subclass analysis showed elevated serum levels of IgG4 subclass. The timeline of the events and treatments is presented in **Figure 1**.

During hospitalization, kidney failure with tubular acidosis was also found. The urine analysis showed microhematuria, proteinuria (1 g/L), and granular casts. The renal ultrasound demonstrated no abnormal findings.

A renal biopsy was performed, and a kidney sample with up to 40 glomeruli was obtained (**Table 1**). The light microscopy showed tubulointerstitial inflammatory infiltrates (mainly composed of lymphoplasmacytic cells), irregular thickening of

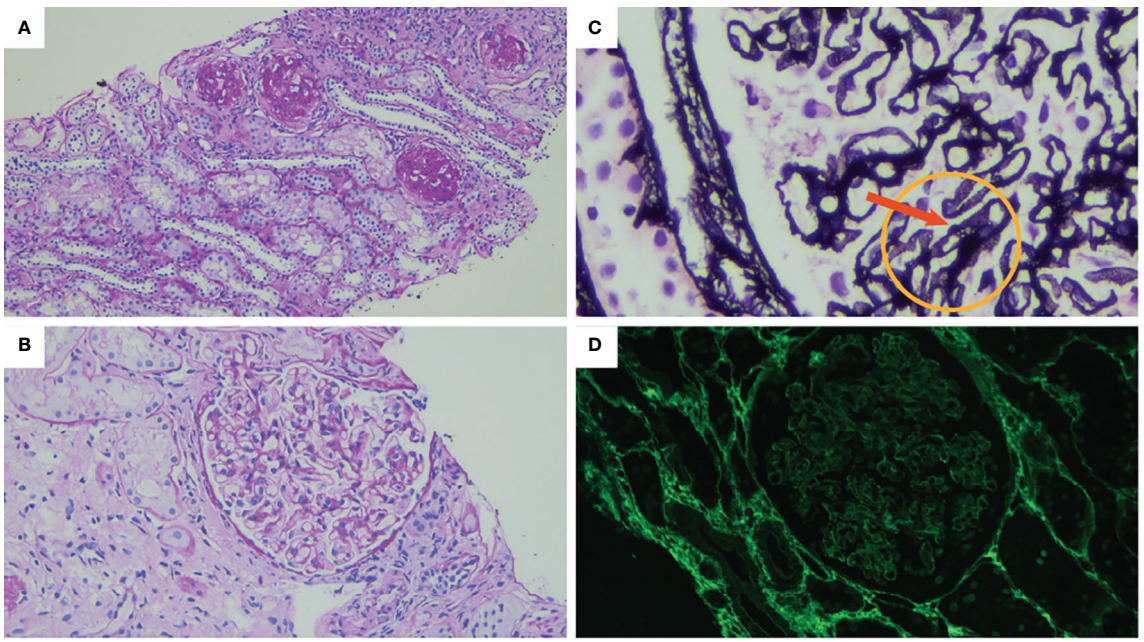

FIGURE 2 | The result of the renal biopsy shows **(A)** diffuse global glomerulosclerosis. Periodic acid Schiff (PAS) stain original magnification: ×100. **(B)** Glomerular basal membrane (GBM) thickening. PAS stain original magnification: ×400. **(C)** Prominent vacuolated appearance of GBM indicated by the arrow. Jones methenamine silver, original magnification: ×1,000. **(D)** No PLA2R immune deposits (immunofluorescence, original magnification: ×400).

the glomerular basement membranes, and subepithelial deposits (**Figures 3A, B**).

Immunofluorescence showed subepithelial glomerular membrane IgG deposits with granular pattern and tubular wall deposits, C3 glomerular deposits, and focal tubular deposits.

A diagnosis of membranous glomerulopathy associated with tubule–interstitial nephritis was made. Immunohistochemical staining for IgG4 demonstrated plasma cells with a complete overlapping positivity for IgG and IgG4 (**Figure 3B**).

IgG–IgG4 Immunohistochemical staining was also performed on BMB, which showed a 50% overlapping positivity for IgG and IgG4. This finding, together with high circulating serum levels of IgG4, led to the definition of an IgG4-related kidney disease (IgG4 RKD).

After the diagnosis of IgG4 RKD, corticosteroid therapy (1 mg/kg/day), targeting both the hematological disorder and glomerulopathy, was started, without clinical response. Thereafter, at 8 months from the onset of the disease, the patient underwent hematopoietic stem cell *transplantation* (HSCT) from his HLA-identical 5-year-old healthy brother. The HSCT characteristics are shown in **Table 2**. During the early period after the infusion of bone marrow cells, the child developed severe gastro-intestinal and cutaneous toxic complications. Engraftment of neutrophils occurred 11 days after transplant, and full donor chimerism (100% donor cells) was demonstrated after the engraftment and confirmed over time. Furthermore, we observed many endothelial complications represented by two episodes of a severe veno-occlusive disease requiring treatment with defibrotide and paracentesis, thrombotic microangiopathy managed with discontinuation of CSA, and cycles of plasmapheresis and eculizumab. The patient developed acute GvHD (a-GvHD) on day +35, with a maximum of grade IV involving the skin, liver, and gastro-intestinal tract with hemorrhagic diarrhea as confirmed by intestinal biopsies. This condition was refractory to steroids at 2 mg/kg, and its management required a high dose of methylprednisolone and etanercept and ileostomy placement for intestinal sub-occlusion. Etanercept and prolonged steroid therapy enable the complete remission of a-GvHD. The evaluation of immunological reconstitution at 1 year after HSCT demonstrated a value of lymphocyte subpopulations within the normal range. After HSCT, the patient experienced severe renal failure triggered by the renal toxic effect of antiviral and antibiotic therapies by septic shocks and by endothelial damage related to transplant. The decrease of renal function required kidney replacement therapy through chronic hemodialysis.

"In consideration of the rarity of the incidence of IgG4 RD in children, we speculated that it could be an epiphenomenon of a hereditary disease. After HSCT, the patient experienced severe clinical issues related to immunosuppression and GvhD that required intensive care and led to a further decrease of renal function, with the need for kidney replacement therapy through chronic hemodialysis. Moreover, in the following years, he presented several complications, such as alopecia, candida infection with hepatic involvement, hyper eosinophilia, hypoparathyroidism, and others.

TABLE 1 | Results of the investigations and comparison between the patients.

	Patient 1	Patient 2
	Hematological and immunological investigations	
	■ Severe thrombocytopenia: 27 × 10^9 L (reference values: 200–450) ■ Latero-cervical and abdominal lymphadenomegaly ■ Vitamine B12: 1,086 pg/ml (reference values: 191–663) ■ DNTs 2.7% (reference values <1.5)	■ Trilinear insufficiency requiring weekly transfusion of red blood cells and platelets ■ Serum IgG4: 353 mg/dl (reference values: <120) ■ BMB: severe hypocellularity (20%); lymphoplasmacytic infiltrate IgG4+ cells/hpf: 0–9 IgG+/IgG4+ ratio: 50% ■ Treg CD4+CD25+FOXP3+
Phenotypic characterization of CD4+FoxP3+CD25 high-Treg lymphocytes (reference values: 1–5%)	2.3%	0.3%
Suppression activity by CD4+CD25 high-Treg lymphocytes (reference values: >25%)	–	17%
	Nephrological investigations	
Kidney biopsy–light microscopy (H&E, PASM, PAS, and Masson's trichrome stains)	Membranous pattern with 7 out of 11 obsolete glomerulus. Active lymphocytic inflammation in tubule–interstitium, especially near arterioles	50% cortex and 50% medulla. Up to 40 glomerulus. Tubulointerstitial inflammatory infiltrate. Irregular thickening of the glomerular basement membranes and subepithelial deposits
Kidney biopsy–immunofluorescence/IHC	Glomerular deposition of IgG and C3. Anti-PLA2R, IgG/IgG4, and THSD7A negative	IgA, IgM, C4 and C1q: negative. IgG and C3 glomerular membrane and tubular deposits IgG deposits. IgG4+ cells/hpf: >10 IgG+/IgG4+ ratio: 100%
eGFR at the time of kidney biopsy (normal values: >90 ml/min)	66 ml/min/1.73 m^2	28.5 ml/min/1.73 m^2
Proteinuria at the time of kidney biopsy (normal values: <0.15 g/24 h)	0.65 g/24 h	1.07 g/24 h
	Next-generation sequencing	
FOXP3 mutation	Leucine-Zipper Domain (exon 8) (NM_014009.3) [c.779T>A (p.L260Q)]	Fork-head domain (exon 11) (NM_014009.3) [c.1087A>G (p.I363V)]

BMB, bone marrow biopsy; DNT, double-negative lymphocytes; H&E, hematoxylin and eosin; IHC, immunohistochemistry; PAS, periodic acid-Schiff; PASM, periodic Schiff-methenamine silver.

In consideration of the above-mentioned clinical manifestations, only partially ascribable to both CKD and GvhD, a further genetic analysis was done. We performed whole-exome sequencing on the proband, using a pre-transplant blood sample, and his relatives (both parents and the asymptomatic brother), using HPO codes addressing also membranous nephropathy and IgG4 RKD. A hemizygous mutation in the Fork-head domain (exon 11) of the *FOXP3* gene (NM_014009.3), c.1087A>G (p.I363V), was found. This variant, inherited from the mother, had not been reported before in the GnomAD database. Nonetheless, it was predicted to be likely pathogenic by the Varsome website and was already described in literature (9); thus, a diagnosis of IPEX was made. Unexpectedly, the same variant was found in hemyzigosis also in the proband's healthy brother, who had been his bone marrow donor. Therefore, we performed a flow cytometry analysis to evaluate the assessment of Treg (CD3+CD4+CD25+Foxp3+) that resulted normal for brother (2.7%) and mother (2.1%) but not in our patient (0.3%). Differently from the exome, a cytometry analysis was performed on post-bone marrow transplant blood sample.

To better characterize the pathogenesis and the kidney involvement of the *FOXP3* mutation in our clinical presentation, we performed indirect immunofluorescence in serum and immunohistochemistry (IHC) on kidney tissue for M-type PLA2R, that resulted highly positive, and THSD7A, that otherwise resulted negative (**Figures 3C, D**).

At the age of 5, the patient underwent deceased donor kidney transplant, and after more than 1 year, he presents normal kidney function and absence of proteinuria. At the age of 7, he was diagnosed with severe maculopathy secondary to multifactorial causes (previous CMV infection, microangiopathy damage, *etc.*), including calcineurine inhibitors. Thus, a therapeutic shift from tacrolimus to everolimus (started with 2 mg/m^2/day) was made. Thereafter, we investigated again the Treg assessment, and we performed T cell proliferation suppression. The percentage of Treg was still low, and Treg suppression activity was significantly lower in our patient compared to that of his sibling (10) (**Supplementary Materials 1–3**).

DISCUSSION

Here we describe two children with *FOXP3* mutations and clinical manifestations mainly represented by variable hematological involvement at the onset, *i.e.*, ALPS in one case and IgG4-related disease in the other, and similar kidney involvement

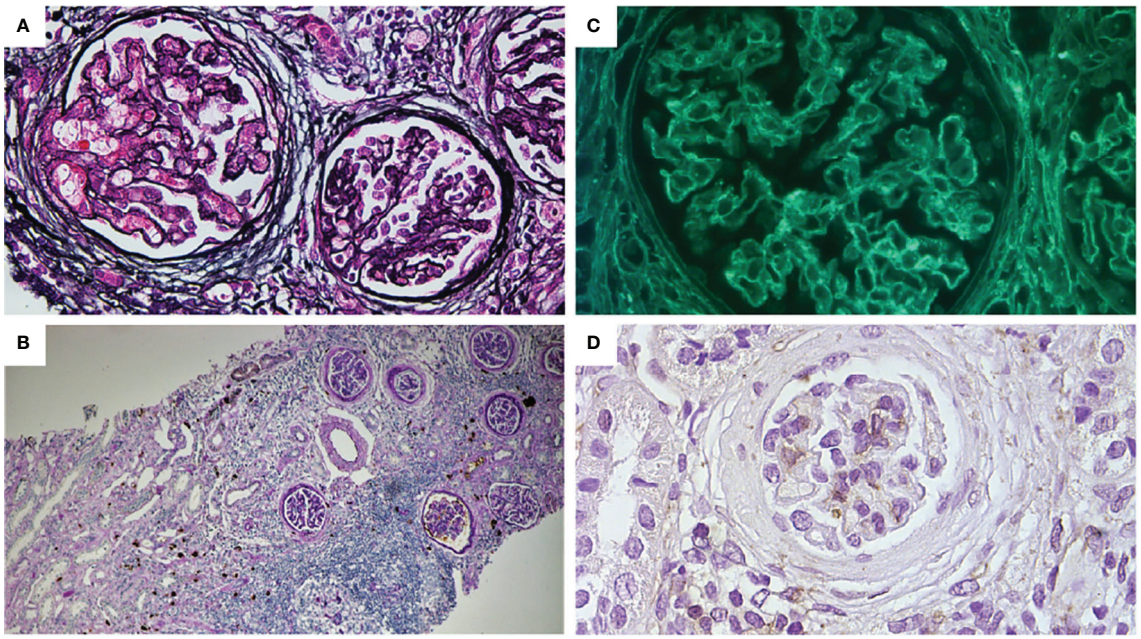

FIGURE 3 | **(A)** Mild thickening of the capillary wall and glomerular basement membrane. Bowman capsule enlargement and reduplication (periodic Schiff-methenamine silver, original magnification: ×400). **(B)** Interstitial and peri-tubular IgG4 deposits. Mild tubular atrophy and interstitial fibrosis. Moderate focal and periglomerular lymphoplasmacytic infiltrate (PAS + IgG4 IHC, original magnification: ×100). **(C)** Glomerular sub-epithelial deposits with granular pattern (immunofluorescence for anti-PLA2R antibodies, original magnification: ×400). **(D)** Absence for THSD7A glomerular expression (IHC original magnification: ×200).

occurring at the second stage of the disease that was membranous glomerulopathy in both cases. Overall, the clinical features associated with *FOXP3* mutations in the two children herein described did not fit a classical diagnosis of IPEX that is usually represented by the classic triad of intractable diarrhea, T1D, and eczema (5) with early-onset, usually under 3-5 years. The two clinical associations here described are therefore anecdotal for the wide spectrum of possible phenotypes associated with *FOXP3* mutations (11).

The first case presented a syndrome with late onset (16 years) in which an ALPS like hematologic condition predominated at the start. The second child presented severe cytopenia due to bone marrow failure at presentation and required weekly platelet and red blood cells transfusions. Molecular and cellular investigations (DEB test, telomere length measurement, etc.) did not allow for a more precise setting in any cBMFs and only highlighted the presence of high IgG4 levels in kidney, bone marrow and in serum. The renal syndrome predominated at a second stage, leading in both cases to the development of chronic renal failure and a similar pathologic involvement with membranous deposits of antibodies (PLA2R positive in only one case). Kidney biopsy features of the two cases are presented and compared in **Table 1**.

ALPS is a rare disorder with immune dysregulation characterized by early-onset, chronic, non-malignant lymphoproliferation, splenomegaly, and autoimmune

TABLE 2 | Hematopoietic stem cell transplantation (HSCT) features and related main complications of patient 2.

Transplant's features	
Main indication to HSCT	Bone marrow failure (failure of first-line treatment with Eltrombopag and GCSF)
Age at HSCT	3 years and 9 months
Stem cell donor	Matched related donor (brother)
Stem cell source–cell dose	Bone marrow - MC 10.7 × 10^8 per kilogram of recipient's weight
Conditioning regimen (cumulative dose)	- Treosulfan 42 gr/m^2 - Fludarabine 160 mg/m^2 - Thiotepa 8 mg/kg - ATG 10 mg/kg
GvHD prophylaxis	- CyA - MMF

ATG, antithymocyte globulin; CyA, cyclosporin A; GCSF, granulocyte colony-stimulating factor; HSCT, hematopoietic stem cell transplantation; MC, mononuclear cells; MMF, mycophenolate mofetil; MPD, methylprednisolone.

manifestations due to defective lymphocyte apoptosis and elevations in CD3+TCRαβ+CD4−CD8− DNTs. Although the diagnosis of ALPS is based on clinical criteria and does not require the presence of any molecular defect (8), ALPS is established to be usually associated with mutations in genes involved in the apoptosis pathway (FAS, FASLG, and CASP10). Moreover, there is growing interest in considering a wide variability of ALPS-like disorders characterized by an expanded number of disease-associated genes (CASP8, NRAS, KRAS, CTLA4, LRBA, FADD, PRKCD, STAT1, STAT3, TNFRSF13B, ADA2, *etc.*) whose mutations result in some overlapping symptoms, including lympho-proliferation, cytopenia, inflammatory bowel disease, malignancy predisposition, and other autoimmune manifestations (12). *FOXP3* could now be added to the list above. Kidney involvement in ALPS is uncommon and rarely described (13–16), with no univocal spectrum of clinical features or histological lesions.

IgG4 RD is a recently recognized systemic immune-mediated disorder with still unclear pathogenesis. It is characterized by fibro-inflammatory tissue damage, IgG-4 positive plasma cells, and often elevated serum IgG4. This systemic disease can potentially affect every organ: pancreas, lymph nodes, lungs, meninges, vessels, kidneys (7). Renal involvement in IgG4 RKD can include tubulo-interstitial nephritis, membranous glomerulopathy (7–10% of cases), and obstructive disorders related to retroperitoneal fibrosis (17, 18). The epidemiology is still poorly described, but the disease appears more frequent in men over 50 years of age, with few cases of IgG4 RD having been reported in pediatric patients (19).

Therefore, the two patients herein described represent unique clinical features associated with pathogenetic *FOXP3* mutations that must be added to the list of clinical syndromes that may occur in this genetic contest. In a minority of cases, IPEX syndrome can indeed present an atypical phenotype and without the classic triad, but the incidence may be underestimated. Overall, our cases are consistent with the increasing evidence of atypical presentations of IPEX and also suggest that clinical manifestations are likely influenced by epigenetic factors or modifying genes (20). The genotype–phenotype correlation in IPEX is not clear: mutations in the DNA-binding site of *FOXP3* seem associated with poor outcomes (21), whereas there are mutations in the Fork-head domain and leucine-zipper domain (12) that are associated more frequently with mild phenotype or late onset. Case 2 is of particular interest in the context of epigenetic modifications since the asymptomatic brother of the proband and HSCT donor presented the same *FOXP3* mutations (unknown at the time of transplantation), thus supporting the evidence that clinical manifestations are unforeseeable, not related to the mutation type, and affected by some not yet identified regulatory mechanisms (22). Moreover, we performed Treg expression analysis on the blood cells of our patient after the hematopoietic stem cell *transplantation*, before and after the shift from tacrolimus to sirolimus, and on the donor in two different timings. The Treg phenotypes of the two brothers were different, and in our patient, the number of Treg was persistently very low, and Treg suppression activity was lower as compared to his sibling after therapeutic shift, strengthening our perspective (**Supplementary Materials 1 and 2**).

Data on kidney disease in IPEX syndrome are scarce: renal involvement is thought to occur in one-third of patients, sometimes as first manifestation of the disease (23). Interstitial nephritis, membranous glomerulopathy, and minimal change disease are the most common forms of renal injury (11).

The variety of kidney alteration in IPEX could be explained by the role of *FOXP3* on regulatory T cells. Tregs have, in fact, functional plasticity in response to different immune and genetic environment (24), and the dysregulation of Tregs could produce two major effects: one is the stimulation of IgG4 autoantibodies *versus* renal-specific antigens (PLA2R1 and THSD7A are the major) in membranous glomerulopathy, while the second potential effect of Tregs dysfunction is the increase of the release of cytokines by effector T cells that affect podocyte function (with the development of minimal change disease) (25). In IPEX syndrome, the pathogenesis of membranous glomerulopathy is consistent, with an imbalance between Treg and Th17 (26) that is due to a significant reduction of Tregs and *FOXP3* expression (27) in the presence of Th17 stable levels.

Parallel activation of the T helper 2 cells (Th2) lineage would promote IgG4 deposition (28). Th17/Treg imbalance may be implicated also in the pathogenesis of IgG4 RD and ALPS that are the two clinical settings herein described in association with FOXP3 mutations. In the former case (IgG4 RD), the increment of T helper 2 cells should stimulate IgG4-producing B cells (29) and upregulate Th17 (30, 31), resulting in a fibro-inflammatory disease involving various organs. Considering some recent evidence on the role of Treg in lymphoproliferative diseases, Mazarolles et al. investigated the Treg profiles in ALPS syndrome and found a reduced expression of CD3+CD4+CD25FOXP3+ Treg subsets (32). Further investigations are needed to support these initial evidence. A final point of interest is therapy. In the young boy with ALPS, immunosuppression was achieved with sirolimus, which spares normal Treg (33) and increases the suppressive function of IPEX patients' Treg cells (34). Sirolimus has been extensively utilized for the control of IPEX syndrome (6), alone or in combination with steroids. This approach was functional to modify the outcome of ALPS and attenuate the clinical presentation of membranous glomerulopathy. In the second case, a steroid therapy was attempted without benefit at the start of symptoms; then, the child underwent HSCT that represents the gold standard for classic and severe forms of IPEX. Our patient had a good relief from the disease after HSCT, but he experienced a severe GvhD that impacted on residual kidney function, leading to the need for chronic kidney replacement therapy. Moreover, some complications experienced by our patients after HSCT (alopecia, candida infection, *etc.*) could be attributed to IPEX syndrome reactivation due to the donor HSCT's FOXP3 mutations. Unfortunately, at the time of HSCT, a genetic diagnosis had not been reached, and in consideration of the urgency of the treatment, the selection of a HSCT donor was made based on available clinical data. With the improvement of massively parallel sequencing

techniques and the reduction of the exam execution times, it will probably become mandatory to screen asymptomatic donors for the family variant prior to transplantation.

Overall, two cases with mutations of *FOXP3* have been described herein that had atypical clinical presentation in comparison to what it is expected in patients with this molecular feature, that is, IPEX syndrome. The renal picture of a primary autoimmune condition, membranous glomerulopathy in both cases, stimulated a genomic analysis that was central to recognize the atypical forms of IPEX. Epigenetic factors may have determined different clinical presentations in patients with classical *FOXP3* mutations, and the description of new phenotypes adds value to the genetic analysis that was central to the definition of the clinical settings. Common mechanisms of regulation of Treg/T helper cells function associated with FOXP3 function could explain the different clinical expressions of these cases that varied from IPEX to IgG4 RD and membranous glomerulopathy. Therapeutic strategies based on drugs modulating Tregs expression positively influenced the clinical outcome of the two patients herein described and should be considered in any other conditions associated with FOXP3 molecular defects.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

MG, ED, AA, LL, and EL had contributed to the conception of the study and wrote the paper. MM, MF, FP, PT, DF, and SG performed hematological and immunological investigations and reviewed the paper. AG, IC, and GC performed genetic analysis and interpretation of the data and reviewed the paper. DV and BC performed immunohistochemical and pathologic evaluation of biopsies. GG, EV, LN, and EF reviewed the manuscript and contributed to the final draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.854749/full#supplementary-material

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CASE REPORT
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Case Report: Use of Obinutuzumab as an Alternative Monoclonal Anti-CD20 Antibody in a Patient With Refractory Immune Thrombocytopenia Complicated by Rituximab-Induced Serum Sickness and Anti-Rituximab Antibodies

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Management of refractory immune thrombocytopenia frequently involves rituximab, a chimeric anti-CD20 monoclonal antibody, to target B cells and induce remission in most patients. However, neutralizing antibodies to rituximab that nullify therapeutic response and may lead to serum sickness have been rarely reported. Here, we present a case of a young adult woman with Evans syndrome treated with rituximab, complicated by the development of serum sickness, acute respiratory distress syndrome, and platelet refractoriness presumed secondary to neutralizing antibodies to rituximab. She was successfully treated with the humanized anti-CD20 monoclonal antibody, obinutuzumab, with subsequent symptom resolution. Additionally, a review of 10 previously published cases of serum-sickness associated with the use of rituximab for idiopathic thrombocytopenic purpura (ITP) is summarized. This case highlights that recognition of more subtle or rare symptoms of rituximab-induced serum sickness is important to facilitate rapid intervention.

Keywords: serum sickness, ITP (idiopathic thrombocytopenic purpura), rituximab, obinutuzumab, case report

INTRODUCTION

Idiopathic thrombocytopenic purpura (ITP) arises from immune clearance or suppression of platelets. Corticosteroids and intravenous immunoglobulin (IVIG) are commonly used in the first-line management of newly diagnosed ITP. However, management of refractory or chronic ITP frequently relies on the use of anti-CD20 monoclonal antibody therapy, most commonly rituximab, a type 1 chimeric IgG antibody (1). Rituximab reversibly depletes CD20+ B cells and induces

remission in 52%–73% of patients with ITP through the cessation of antibodies directed against platelet-surface glycoproteins (2). Relapse of ITP is common; however, retreatment is often successful, as 80% of patients respond to repeat rituximab courses (3).

In general, rituximab is well-tolerated apart from a common first-dose infusion reaction that is primarily due to rapid cytokine release because of brisk destruction of B-cell targets by the monoclonal antibody. Infusion reactions should not be confused with the rarer type III immune-complex-mediated hypersensitivity reaction that may occur from anti-rituximab antibodies and often results in rituximab-induced serum sickness (RISS). Prevalence of RISS is reported at high rates in patients with systemic autoimmune disorders, as high as 39% in patients with systemic lupus erythematosus (4). In children with ITP, the prevalence is lower, reported to be between 6% and 12% (5, 6). RISS may often be under-recognized, especially with earlier infusions, as less than half of patients present with the classic triad of fever, rash, and arthralgias (7).

Prompt recognition of RISS and initiation of corticosteroids are important in the management of ITP patients, particularly as re-exposure to rituximab is common and may trigger more severe clinical manifestations such as anaphylaxis (8). Newer humanized (e.g., obinutuzumab) and fully human (e.g., ofatumumab) monoclonal anti-CD20 antibodies exist that may have less risk of serum sickness without cross-reacting with rituximab but have rarely been employed in the treatment of ITP (9).

Here we report a 25-year-old patient treated with rituximab complicated by the development of serum sickness, acute respiratory distress syndrome (ARDS), and platelet refractoriness presumed secondary to neutralizing antibodies to rituximab successfully treated with obinutuzumab. Additionally, a review of 10 previously published cases of serum sickness associated with the use of rituximab for ITP is summarized.

CASE DESCRIPTION

A 25-year-old woman with relapsing–remitting Evans syndrome presented with refractory severe thrombocytopenia and grade III mucosal bleeding despite prednisone, intravenous IVIG (1 g/kg × 3 doses), romiplostim (10 μg/kg), and rituximab. Her CD20+ B-cell counts remained normal despite 100 mg/m^2 × 3 doses and 375 mg/m^2 × 2 doses of rituximab. Eighteen days after her first rituximab dose, she reported new-onset severe neuropathic pain in her right leg diagnosed as piriformis syndrome. Subsequently, she developed fevers, malaise, arthralgias, blurry vision, and abrupt acute hypoxic respiratory failure with intracranial hemorrhages requiring mechanical ventilation (**Figure 1**). While her thrombocytopenia was associated with petechiae, no other discrete rash was observed. Her arthralgias began 5 days after her third rituximab dose, fevers started 17 days after her fifth rituximab dose, and respiratory symptoms developed 18 days after her fifth rituximab dose. Extensive evaluation for infectious etiologies of her fever and ARDS was negative. Malignancy screening, including a bone marrow biopsy, was negative for lymphoproliferative disorders. Additionally, further evaluation with whole-exome sequencing for underlying inborn errors of immunity and screening for systemic autoimmune disorders was non-diagnostic. Of note, she was previously treated with rituximab 375 mg/m^2 × 4 doses four years prior for ITP without incident. However, repeat dosing for an ITP relapse one year prior with rituximab 100 mg/m^2 × 4 doses was complicated by an infusion reaction with her initial dose (bronchospasm requiring treatment with hydrocortisone,

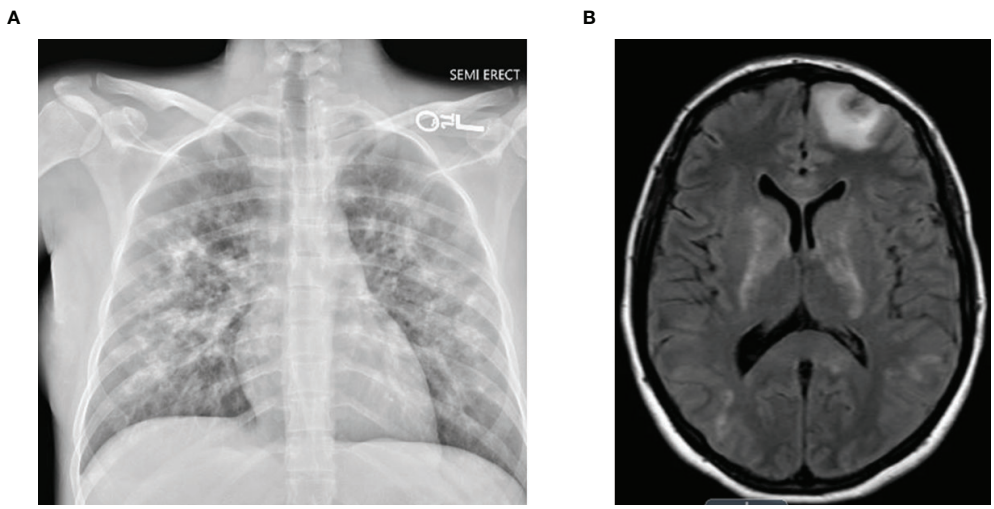

FIGURE 1 | **(A)** Chest X-ray revealing diffuse interstitial and airspace opacities. **(B)** Brain MRI revealing extensive fluid-attenuated inversion recovery (FLAIR) signal abnormalities throughout supratentorial parenchyma.

famotidine, and albuterol). She also reported fatigue and jitteriness following her third and fourth doses that improved with corticosteroids with early B-cell recovery within 2 months.

Suspecting neutralizing anti-rituximab antibodies, which were later confirmed [LapCorp rituximab drug level <0.2 μg/ml, anti-rituximab antibodies 4,502 mg/ml (normal < 25)], she received two doses of obinutuzumab 1,000 mg/m^2 with rapid depletion of her CD20+ B cells. Given the severity of her bleeding, she was concurrently retreated with IVIG, continued on high-dose corticosteroids and romiplostim, and initiated on mycophenolate mofetil that was subsequently transitioned to sirolimus (**Figure 2**). She had gradual resolution of her thrombocytopenia. Her ARDS improved following administration of obinutuzumab, and her severe neuropathic pain, despite being previously refractory to multitherapy treatment with gabapentin, cyclobenzaprine, and opioids, improved following obinutuzumab administration coincident with her platelet count stabilizing. Since multiple therapeutic interventions were initiated concurrently, we are unable to definitely determine which agent led to improvement. There was a decrease in B-cell count following mycophenolate mofetil treatment; however, the abrupt and complete elimination of B cells did not occur until after obinutuzumab administration. She remains on sirolimus with intermittent doses of romiplostim to maintain a platelet count >50,000 cells/μl and has documented the repopulation of her CD20+ B cells.

DISCUSSION

While more commonly reported in systemic autoimmune disease, RISS remains a rarely reported complication of therapy in ITP despite the broad use of rituximab as therapy for chronic or refractory ITP (**Table 1**). Details for some of the previously published cases are sparse, but cases of infants through adults (8 months to 48 years) are reported. As shown (**Table 1**), 7/8 (87.5%) ITP patients with available symptom data were documented to have the classic triad symptoms of fever, rash, and arthralgias. Malaise and fatigue were also reported in 5/8 (62.5%) ITP patients with available symptom data.

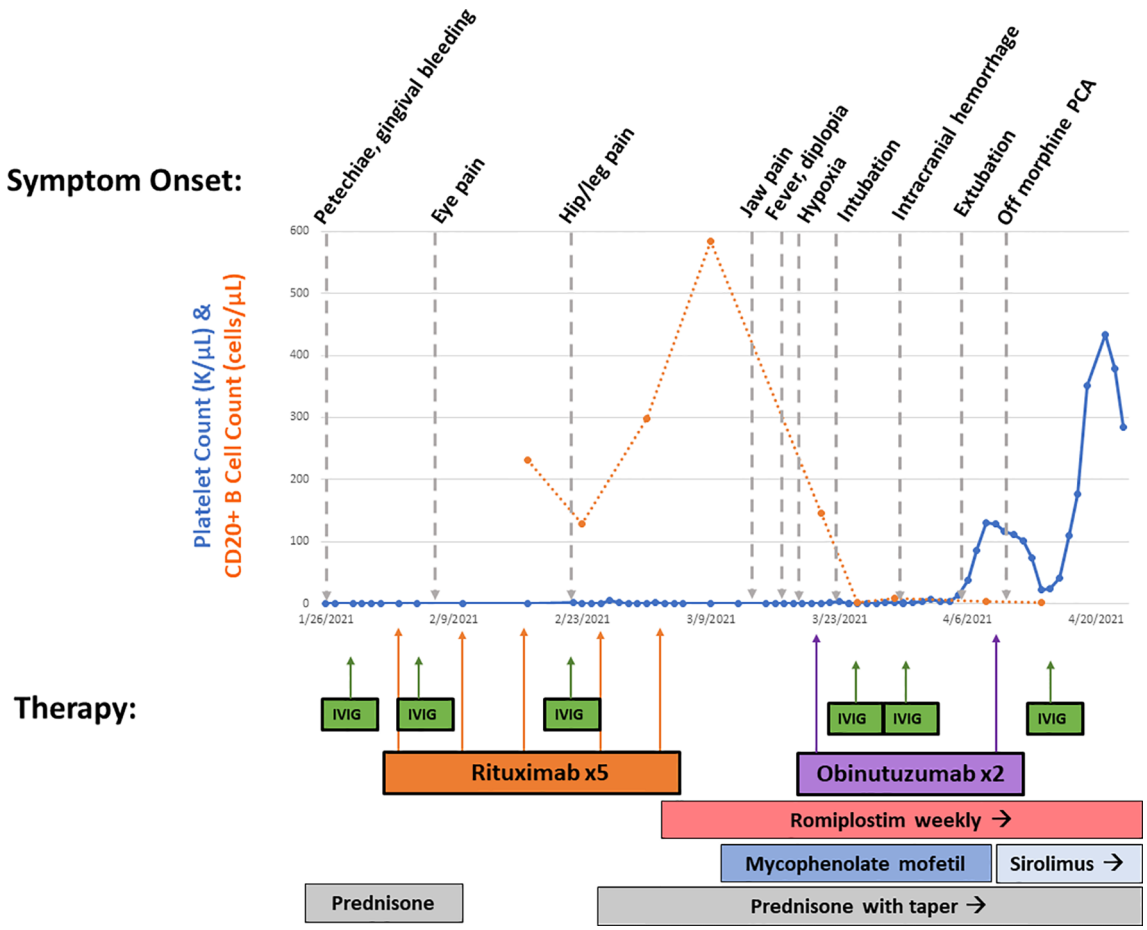

FIGURE 2 | Overview of treatment course. Platelet count (in K/μl, solid line) and CD20+ B-cell count (in cells/μl, dotted line) from beginning of presentation until hospital discharge is shown. Symptom onset is shown on top, while therapies are shown on bottom with corresponding arrows. This demonstrates rapid B-cell depletion following obinutuzumab therapy with gradual rise in platelet count.

TABLE 1 | Previously published case reports of rituximab-induced serum sickness in patients with immune thrombocytopenia.

Publication	No. pts.	Age/ gender	No. doses	Concurrent diagnoses	Presence of anti-rituximab antibodies?	Classic symptoms			Other features	RISS treatment?
						Fever?	Rash?	Arthralgias?		
Godeau et al. (10)	1	NA[§]	2	NA	NA	NA[§]	NA	NA	No	None
Wang et al. (6)	3	14F	2	NA	NA	Yes	Yes	Yes	Malaise	NA
		12F	3	NA	NA	No	No	Yes	Rash during dose 1	NA
		12F	1	NA	NA	Yes	Yes	Yes	Malaise	NA
Bennett et al. (5)	2	12M	2	NA	NA	Yes	Yes	No	Fatigue	NA
		11F	2	NA	NA	Yes	Yes	Yes	Conjunctival hyperemia	NA
Goto et al. (11)	1	8M	2	No	Yes	Yes	Yes	Yes	Fatigue and rash developed 11 days after fever, arthralgias	Prednisolone
Medeot et al. (12)	1	NA[¶]	2	NA	NA	NA[¶]	NA	NA	No	None
Herishanu et al. (13)	1	48F	2	No	NA	Yes	Yes	Yes	Malaise	Methyl-prednisolone
Manko et al. (14)	1	46F	1	Asthma	NA	Yes	Yes	Yes	Hypoxemic respiratory failure (ARDS)	IVIG + plasmapheresis

NA, not available; RISS, rituximab-induced serum sickness; ARDS, acute respiratory distress syndrome; IVIG, intravenous immunoglobulin.

[§]Information not available, but in an adult cohort (18–84) and listed symptoms as transient serum sickness.

[¶]Information not available, but in an adult cohort (18–76) and listed as grade 3 serum sickness with rapid improvement.

Clinically, it is important to differentiate between the more commonly observed rituximab-related infusion reaction and serum sickness, as each occurs due to a unique immune mechanism, therefore requiring different management approaches. Infusion reactions are primarily noted with the first infusion and are more commonly reported in patients with hematologic malignancies as compared to those with autoimmune conditions (15). Patients often report fever, chills, rigors, pruritus, nausea, headache, or less commonly hypotension, hypoxia, and bronchospasm. Typically, infusion-related reactions are secondary to cytokine release (15) and can be managed with acetaminophen, antihistamine, and corticosteroids as needed or as pre-medications. RISS, however, generally develops 1–2 weeks after exposure to the offending agent and may be within a few days of subsequent doses. The long half-life of rituximab also increases the risk of late symptoms occurring. Serum sickness occurs when excess non-human or heterologous antigens, such as murine Fab fragments of rituximab, bind to circulating IgG antidrug antibodies and form immune complexes. This occurs more commonly with chimeric antibodies, which contain more foreign antigens than humanized or human antibodies (16). Generally, the intermediate-sized immune complexes that deposit in vessel walls and tissues result in the activation of complement, granulocytes, and macrophages that trigger inflammation, increased vascular permeability, and tissue damage. The most frequently observed symptoms include fever, rash, and arthralgias, although less common symptoms include headache/blurry vision, edema, lymphadenopathy, splenomegaly, peripheral neuropathy, nephropathy, and/or vasculitis (17). Hypocomplementemia is also frequently observed in serum sickness (17), and in fact, the C4 complement level was low in our patient, but C3 was normal (C4 10 mg/dl, C3 147 mg/dl). Additionally, the development of ARDS in the case reported by Manko et al. (14), as well as in our patient, is postulated to be a consequence of immune-complex deposition leading to alveolar damage and vascular leak. While mild cases can be managed with non-steroidal anti-inflammatory drugs and/or antihistamines, severe cases require corticosteroids and consideration of IVIG and/or plasmapheresis (14).

When RISS occurs, it prompts the decision to stop therapy; however, it may be possible to change to the humanized type II anti-CD20 antibody, obinutuzumab, as used in our patient, or to the human type I antibody, ofatumumab (18). Since rituximab and ofatumumab are both type I antibodies, they rely more on complement-dependent cytotoxicity (CDC) with ofatumumab notably engineered to be even more dependent on CDC (19). Complement is often depleted from consumption with type III hypersensitivity reactions. Thus, we chose obinutuzumab for treatment, as it is a type II antibody that is glycoengineered to work primarily through antibody-dependent cellular cytotoxicity (ADCC) and direct cytotoxic mechanisms. Theoretically, there could be a slightly higher potential of cross-reactivity with rituximab and the humanized antibody. However, one small report did not see a difference between the drugs (19). Unfortunately, even fully human antibodies, which have no mouse component, may be immunogenic, especially in patients with autoimmune disorders that may have a defect in tolerance mechanisms (20).

Given the rarity of RISS in ITP patients, it is critical to maintain a high index of suspicion, particularly in higher-risk patients such as those receiving multiple courses of rituximab and those with an underlying systemic autoimmune disease. Perhaps even more problematic is the presence of neutralizing anti-rituximab antibodies, which may not elicit an immune response and result in a faster B-cell reconstitution or, as in this case, no clearance of B cells. Neutralizing antibodies may contribute to the 20%–40% non-response or loss of response to rituximab in many disorders. This poses the question as to whether an assessment of rituximab activity should be routinely monitored. While possible to measure anti-rituximab antibodies, it generally requires samples to be sent out, and the turnaround time may limit practical utility. Flow cytometry to measure B-cell levels is more accessible but less specific. While

rituximab has become a routine agent in patients for non-oncologic purposes, it is critical that recognition of more subtle or rare symptoms of RISS is appreciated to facilitate rapid intervention, most importantly drug discontinuation, and to either try desensitization protocols or prompt transition to alternate therapy for the underlying disorder (21).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JB and KW: conception and design. JB, DF, TM, and KW: writing, review, and revision of the manuscript. All authors read and reviewed the manuscript and contributed to and approved the final version as submitted.

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MINI REVIEW
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Deficiency of Human Adenosine Deaminase Type 2 – A Diagnostic Conundrum for the Hematologist

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Deficiency of adenosine deaminase type 2 (DADA2) was first described in 2014 as a monogenic cause of polyartertitis nodosa (PAN), early onset lacunar stroke and livedo reticularis. The clinical phenotype of DADA2 is, however, very broad and may involve several organ systems. Apart from vasculitis, children may present with i) Hematological manifestations (ii) Lymphoproliferation and iii) Immunodeficiencies. Patients with DADA2 can have variable patterns of cytopenias and bone marrow failure syndromes. Patients with DADA2 who have predominant haematological manifestations are associated with ADA2 gene variants that result in minimal or no residual ADA2 activity. Lymphoproliferation in patients with DADA2 may range from benign lymphoid hyperplasia to lymphoreticular malignancies. Patients may present with generalized lymphadenopathy, splenomegaly, autoimmune lymphoproliferative syndrome (ALPS) like phenotype, Hodgkin lymphoma, T-cell large granular lymphocytic infiltration of bone marrow and multicentric Castleman disease. Immunodeficiencies associated with DADA are usually mild. Affected patients have variable hypogammaglobulinemia, decrease in B cells, low natural killer cells, common variable immunodeficiency and rarely T cell immunodeficiency. To conclude, DADA2 has an extremely variable phenotype and needs to be considered as a differential diagnosis in diverse clinical conditions. In this review, we describe the evolving clinical phenotypes of DADA2 with a special focus on haematological and immunological manifestations.

Keywords: deficiency of human adenosine deaminase type 2, haematological abnormalities, inborn errors of immunity (IEIs), lymphoproliferation, bone marrow failure syndromes, cytopenia

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1 INTRODUCTION

Deficiency of adenosine deaminase 2 (DADA2) is a multifaceted autosomal recessive autoinflammatory syndrome. It is caused by loss-of-function homozygous or compound heterozygous variants in *ADA2* (*adenosine deaminase 2*) gene, formerly named as *CECR1* (*cat eye syndrome chromosome region, candidate gene 1*) (1, 2). Initial descriptions of this disorder were published in 2014 with early-onset stroke, recurrent bouts of inflammation, and familial vasculopathy resembling polyarteritis nodosa (1, 2). Phenotypic descriptions of DADA2 have been expanded considerably and now include vasculopathy, lymphoproliferation, immunodeficiency and bone marrow dysfunction. The large phenotypic variability

makes DADA2 a true multisystemic and multifaceted disorder. It is possible that several other phenotypic presentations of DADA2 is due for recognition in coming future.

Hematological presentations of DADA2 including immune cytopenias and lymphoproliferation (both benign as well as lymphohematopoietic neoplasms) are increasingly being recognized (3). Establishing a diagnosis of DADA2 in patients with hematological disorders is imperative due to immense therapeutic and prognostic implications. We herein review the diverse clinical spectrum of DADA2 with special focus on hematological manifestations.

2 PATHOPHYSIOLOGY OF ADA DEFICIENCY

In humans, two types of partially homologous adenosine deaminase (ADA) enzymes (ADA1 and ADA2) regulate purine metabolism, converting adenosine/2'-deoxyadeosine to inosine/2'-deoxyinosine. While ADA1 is monomeric and predominantly intracellular, ADA2 is the secreted isoform which also exists as dimers.

2.1 ADA1 Deficiency

Though ADA1 is expressed in all human tissues, maximal expression is noted in lymphocytes and is critically important for development of adaptive immune system (4–6). Deficiency of ADA1 results in severe combined immunodeficiency with profound depletion of T, B, and NK cells due to accumulation of toxic deoxyadenosine nucleotides.

2.2 ADA2 Deficiency

ADA2 is highly expressed in myeloid cells and is secreted by activated macrophages, monocytes and dendritic cells (7–9). ADA2 also interacts with lymphocytes and other leukocytes through adenosine receptors (10). ADA2 plays a significant role in development of hematopoietic and endothelial cells, maintaining balance between M1 and M2 macrophages (7, 9, 11). Pathophysiology of DADA2 is still evolving. In DADA2, monocyte differentiation is skewed towards pro-inflammatory M1 macrophages and results in generation of inflammatory cytokines like interleukin (IL)-6 and tumour necrosis factor (TNF)-α (3). Nihira et al. had performed transcriptomic and proteomic analysis on peripheral blood mononuclear cells in a Japanese cohort of DADA2 patients and reported elevated type II interferon signatures. By network analysis, the authors identified *STAT1 gene* as pivotal gene in pathogenesis of DADA2. Further, STAT1 phosphorylation in monocytes and B cells following interferon gamma stimulation was significantly higher in patients with DADA2 as compared to controls (12). Watanabe et al. performed single cell RNA sequencing in monocytes (CD14+) from DADA2 patients and healthy controls. They confirmed higher numbers of non-classical monocytes and an up regulation of M1 macrophage markers in DADA2 patients. Thus, authors suggested that high levels of IFNγ may drive the differentiation of monocytes to a M1 phenotype that leads to release of proinflammatory cytokine TNFα (13).

3 SPECTRUM OF CLINICAL MANIFESTATIONS

Since the initial description, vasculitis/vasculopathy has been the predominant phenotype seen in DADA2. However, a myriad of clinical manifestations are increasingly being reported.

3.1 Vasculopathy

Vasculopathy, affecting the medium and small sized arteries, is the commonest manifestation of DADA2. Clinical manifestations vary from limited cutaneous involvement to severe and fatal systemic vasculitis with multiorgan involvement. Cutaneous involvement includes livedoid rash, erythema nodosum, peripheral gangrene, ulcers and Raynaud's phenomenon. Most common cutaneous manifestation of DADA2 is livedo racemosa. Skin biopsy often shows extensive neutrophil infiltration predominantly in interstitium, macrophage infiltration, and perivascular T lymphocytes without overt features of vasculitis.

In systemic involvement, central nervous system (CNS) is most commonly involved followed by renal and gastrointestinal (GI) systems. Hallmark of CNS vasculopathy is recurrent ischemic lacunar strokes. Other CNS manifestations include cranial nerve palsy, spastic diplegia, encephalopathy, peripheral neuropathy, sensory neural hearing loss, labyrinthitis, and cerebral atrophy. Although abdominal pain and inflammatory bowel disease are predominant GI manifestations, intestinal perforation and aneurysms in celiac and mesenteric arteries have also been reported (14). Renal involvement in patients with DADA2 is seen in form of renal artery stenosis, renal artery aneurysms, arterial hypertension, and glomerular scarring (1, 15, 16).

3.2 Immunodeficiency

The initial reports of DADA2 describe this disorder as a mild immunodeficiency with reduced levels of immunoglobulin (Ig) M. Subsequently, immunological aberrations in DADA2 are being reported in a much greater detail. Up to two-thirds of patients with DADA2 may have decreased levels of either Ig isotypes, while hypogammaglobulinemia has been reported in approximately one fourth of patients (17). Impaired vaccine responses have also been reported (18). Around 10% of these patients present with B cell lymphopenia and low switched memory B cells. Overt immunodeficiency in the form of recurrent infections has been noted in 15–20% of patients (19). Clinical presentation of immunodeficiency phenotype may mimic common variable immunodeficiency (CVID). Therefore, it is prudent to consider differential diagnosis of DADA2 in patients with CVID-like immunodeficiency especially having vasculopathic manifestations (20–22). As recurrent inflammation in DADA2 may inhibit B cell differentiation and function, treatment with anti-inflammatory therapy may be beneficial in improving Ig levels (21). Tissue biopsies can also show the CVID-like phenotype with absent plasma cells (23). Besides CVID-like presentation, clinical features suggestive of a combined immunodeficiency have also been noted in DADA2. These patients have been reported with fungal infections and

infections from DNA viruses including molluscum contagiosum, warts, and members of herpes virus family (24). Low numbers of natural killer (NK) cells and T cells have also been reported (18, 25). Overall, lymphopenia is reported in 15% of patients with DADA2 (17). A recent report on DADA2 patients using in-depth immunophenotyping and functional analysis of lymphocytes revealed a multitude of immunological aberrations. These include impaired class switching and differentiation of B cells, reduced memory and regulatory T cells, increased senescent T cells, diminished mucosa associated invariant T cells and invariant NKT cells, and decreased classical monocytes (26). The authors also reported arrest in B cell development in the bone marrow at the pro-B to pre-B cell stage and defect in terminal B cell differentiation. Authors have also shown that healthy heterozygous carrier state for DADA2 showed intermediate values of lymphocyte phenotypes and functions in comparison to DADA2 patients and healthy controls, further highlighting the role of heterozygous state (26). Thus, immune defect in patients with DADA2 deficiency may present with myriad immunological aberrations (26). Immunological abnormalities reported in patients with DADA2 have been summarized in **Figure 1**.

3.3 Hematological Manifestations of DADA2

3.3.1 Bone Marrow Failure

Bone marrow hypofunction resulting in erythroblastopenia, leukopenia/neutropenia and thrombocytopenia was reported since the first descriptions of DADA2 (1). Since then, pure red cell aplasia (PRCA) or Diamond-Blackfan anemia (DBA)-like presentation is increasingly being recognized in patients with

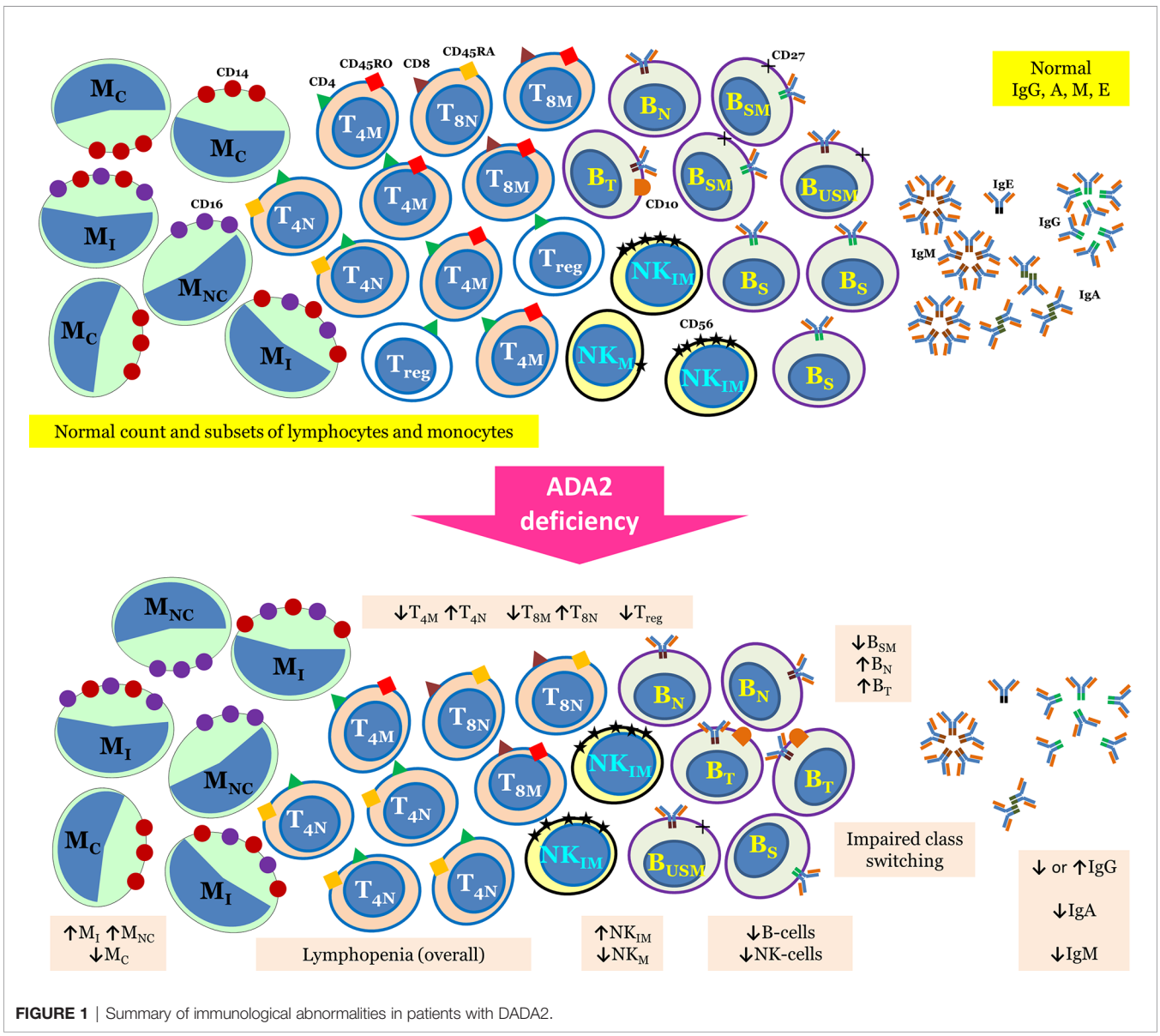

FIGURE 1 | Summary of immunological abnormalities in patients with DADA2.

DADA2. PRCA, as the name suggests, is characterized by absence (or near absence) of red cell precursors from the bone marrow. It manifests as normocytic normochromic anemia with marked reduction in the reticulocyte response (27). DBA represents one of the congenital forms of PRCA that usually presents early in life with macrocytic (occasionally normocytic) normochromic anemia (27, 28). To date, more than 3 dozen such patients with DADA2 have been reported in the literature with DBA (29–36). As DADA2 is not commonly considered amongst the differentials of PRCA/DBA, this leads to significant diagnostic delay in some cases (36). Moreover this disorder has been described recently and possibly many cases of DBA due to DADA2 have been underreported. Majority of DADA2 patients with PRCA/DBA-like clinical features have at least one of the following clinical features, such as, benign lymphoproliferation (lymphadenopathy and hepatosplenomegaly), low IgM/IgA/IgM, or reduced B/NK/CD4-T/CD8-T cells, with or without recurrent or unusual infections. Few patients may also present with stroke and livedo reticularis which can be attributed to vasculitis or vasculopathy (29, 36–38).

Pathophysiologically, classical DBA, resulting from mutations in genes involved in ribosomal biogenesis, is associated with impaired rRNA maturation, while DBA-like illness associated with DADA2 is not (28). Although the precise mechanisms remain unknown, elevated erythroid ADA enzyme activity (especially ADA1) is seen in up to 90% patients with classical forms of DBA (28). In contrast, DADA2 is associated with normal erythrocyte ADA enzyme activity (with reduced plasma ADA2 activity) (39). In routine clinical practice, normal mean corpuscular red blood volume (MCV) in patients with DBA-like illness favours diagnosis of DADA2 over classical DBA. Besides, lack of congenital malformations, seen in ~50% of patients with classical forms of DBA, may also serve as a clue for diagnosis of DADA2 (28).

In addition to erythroid hypofunction, DADA2 may also involve other bone marrow cell lines. Of these, neutropenia is more commonly recognized (up to 10%) and has been described in numerous reports (32, 33, 40–43). Transient neutropenia has also been reported (42). Low IgM or pan-hypogammaglobulinemia, lymphopenia (40–43) has also seen in this subgroup of patients. Other than bone marrow failure syndrome manifestations recurrent fevers, oral ulcers, recurrent infections (including warts) have been reported in this subgroup of patients (32, 41–43). Besides neutropenia, a recent report from NIH on 60 patients with DADA2 describes thrombocytopenia and pancytopenia in 10% of patients (44).

ADA2 has been known to itself act as a growth factor (7, 9) with a potential to modulate secretion of other growth factors as well. Contemporary evidence also suggests both ADA1 and ADA2 enzymes to play a crucial role in development of progenitor cells in the bone marrow (45, 46). The precise contributions of growth factor or ectonucleotidase properties of ADA2 towards development of marrow cells remains to be delineated, resulting in significant gaps in understanding of pathogenesis of marrow hypofunction. A possibility of immune-related marrow hypofunction (as a result of autoimmunity) also exists (17, 46). Besides 'central' (i.e. marrow hypofunction) cytopenias, 'peripheral' cytopenias (e.g. autoimmune) may also can occur in patients with DADA2. This overlap of 'central' and 'peripheral' cytopenias may pose a significant diagnostic and therapeutic challenge for clinicians.

3.3.2 Immune Cytopenias

Autoimmune cytopenia is a common presentation of DADA2. To date, more than a dozen such patients of DADA2 have been reported accompanied with predominant autoimmune cytopenia. In most of these patients the clues for etiological diagnosis have ranged from vasculopathic ulcers (30), stroke (1), low IgG/IgM or hypogammaglobulinemia (32, 44–48), recurrent infections (18, 49) including the vaccine pathogens (46). Lymphopenia, neutropenia, thrombocytopenia (Evans syndrome), lymphoproliferation with/without elevated double negative T-cells (raising a possibility of autoimmune lymphoproliferative syndrome) has also been reported in these patients (1, 18, 30, 32, 46, 49–51). In addition to expected bone marrow examination findings of erythroid hyperplasia (with reticulocytosis), features of erythroid hypoplasia or dysplasia (with reticulocytopenia) have also been described in these patients (46, 49, 50). Direct antiglobulin test was also positive in absence of overt hemolysis and PRCA in few patients (50, 52). Concomitant occurrence of AIHA and erythroblastopenia, hence, seems to be an additional haematological clinical presentation of DADA2.

3.3.3 Other Haematological Manifestations

In a recent report, arthritic presentation mimicking systemic juvenile idiopathic arthritis has also been reported in DADA2 (53). Features of macrophage activation syndrome/hemophagocytic lymphohistiocytosis (MAS/HLH), haemolytic anemia (non-immune), and persistent cytopenias (with a hypercellular bone marrow) were noted in this patient (53). MAS/HLH was also been reported in the first descriptions of this disease, besides many published and unpublished observations (1, 26, 38).

3.4 Lymphoproliferation in DADA2

3.4.1 Benign Lymphoproliferation

Benign lymphoproliferation, resulting from follicular hyperplasia and manifesting as hepatosplenomegaly and/or lymphadenopathy, is a well-recognized feature of DADA2 and is seen in about a third of all cases (3, 17). Idiopathic Castleman disease with benign lymphoproliferation as one of the cardinal clinical manifestations has also been reported in patients with DADA2 (37, 54). Besides, EBV driven non-malignant (benign) proliferation has also been reported in DADA2 (42, 52).

DADA2 has also been reported to present with autoimmune lymphoproliferative syndrome (ALPS) like phenotype (46, 51, 55) given the occurrence of both benign lymphoproliferation and autoimmune cytopenias in this disease. Differentiating DADA2 from classical ALPS may be very difficult. Patients with DADA2 are unlikely to fulfill the primary 2009 NIH ALPS criteria (i.e.,

patients with DADA2 would have normal apoptosis assays and lack the relevant *FAS*, *FASL*, or *CASP10* variants) (55). Besides, presence of lymphopenia and hypogammaglobulinemia favours the diagnosis of DADA2 over classical ALPS (46). Other potential subtle clues would include normal (51) (or mildly elevated) (46, 55) double-negative T-cells and normal (46) or mildly increased (55) levels of vitamin B_{12} (well below 1500 pg/mL) in patients with DADA2. Recognition of other clinical features reminiscent of DADA2 (e.g. vasculopathy/vasculitis) may also help to clinch the diagnosis.

3.4.2 Malignant/Neoplasms

Malignant lymphoproliferation or neoplasms are rare in DADA2. To date, less than a dozen such patients have been reported in the literature. T-cell large granulocytic lymphocytic infiltration/leukemia (T-LGL I/L) has been reported in 2 patients (18). Both these patients also had AIHA and organomegaly concomitantly or prior to diagnosis of T-LGL I/L. Besides, decreased numbers of plasmablasts, transitional B cells, and total switched memory B cells with an increase in activated B and CD4 T-cells (HLA-DR^+), CD8 effector memory RA cells were noted in both patients. Autoimmune neutropenia, thrombocytopenia, pan-hypogammaglobulinemia, and recurrent infections were also described during the clinical course in one of the above patients (18). Hodgkin lymphoma (HL) has been reported in 4 patients with DADA2 including a sibling pair (51, 56–58). In the siblings with HL and DADA2, lymphopenia and hepatosplenomegaly were noted before and hypogammaglobulinemia after initiation of chemotherapy for HL. Both these patients had positive anti-EBNA (Epstein-Barr virus nuclear antigen) IgG but negative anti-EBNA IgM (57). In the other 2 patients, arthritis, vasculopathy, and neutropenia were additional features of DADA2 (57, 58). Cutaneous acute myeloid leukaemia (AML) (30) and diffuse large B cell lymphoma (DLBCL) (with high EBV viral load) have also been reported in patients with DADA2 (33, 59).

4 DIFFERENTIAL DIAGNOSIS

As we have summarized, DADA2 is associated with a myriad of clinical manifestations. Hence, this disorder would be an important differential for a variety of illnesses including vasculitis/vasculopathy, inborn errors of immunity (especially humoral immunodeficiencies) including immune dysregulatory (e.g. recurrent fevers, HLH) and lymphoproliferative disorders (e.g. ALPS), PRCA/DBA, marrow dysfunction/pancytopenia, autoimmune cytopenias, and occasionally neoplasms. Although DADA2 might present with single system involvement, presence of personal or family history of relevant multisystem manifestations (e.g. lymphoproliferation and vasculitis/vasculopathy, cytopenias and hypogammaglobulinemia, and other combinations) may, by far, be the simplest clinical clue for its diagnosis.

5 LABORATORY DIAGNOSIS OF DADA2

5.1 Quantification of ADA2 Enzymatic Activity

ADA2 activity can be measured by spectrophotometry or LC-MS/MS based assay in serum/plasma/tissue-culture supernatant or dried plasma spot respectively. It quantifies the adenosine-dependent generation of ammonia in the presence of erythro-9-Amino-β-hexylα-methyl-9H-purine-9-ethanol hydrochloride (EHNA), a selective inhibitor of ADA1. It is essential to perform ADA enzyme activity in addition to genetic analysis since heterozygous carriers can present with decreased enzymatic activity and clinical manifestations (38). Newer cost-effective and rapid methods for estimation of ADA2 enzyme activity may serve as a screening tool for ordering genetic testing in patients with DADA2. Utilizing such techniques, Cafaro et al. noted all patients with variant-proven DADA2 to have ADA2 enzyme activity of ≤0.06 mU/mL (60).

5.2 Genetics

Genetic sequencing remains the mainstay of genetic diagnosis of DADA2. Next-generation sequencing (NGS) including whole-exome sequencing is increasingly being utilized to diagnose DADA2, even when assays for ADA2 enzyme activity are not readily available (61). Given the pleiotropic manifestations of DADA2, it is important to include *ADA2* gene in various customized NGS panels used for evaluation of haematological, immunological, and rheumatological disorders. As the most common disease variants are found in exon-2 (p.G47R, p.G47A) followed by exon 3 and 4 (**Supplementary Table**), Sanger sequencing can also be employed upfront for evaluation. Few *ADA2* pathogenic variants (homozygous 800 bp duplication in exon 7), however, may not be detected by such strategies. Other techniques such as Multiplex Ligation-dependent Probe Amplification (MLPA) in combination with long-read polymerase chain reaction (PCR) sequencing need to be employed in such scenarios (55). Deep RNA sequencing can be used to evaluate the impact of novel splice variants (62).

6 PROFILE OF PATHOGENIC VARIANTS IN *ADA2* GENE

Missense, nonsense, splice site mutations, frame shift mutations, deletions, and copy number variations have been documented in the ADA2 protein with maximum clustering seen in the catalytic domain (**Figure 2** and **Supplementary Figure 1**). The most commonly reported mutations in different ancestries are p.Gly47Arg (Asian, Georgian-Jewish, Turkish), p.Gly47Ala (European Caucasian), p.Arg169Gln (European Caucasian, Dutch, Belgium, and Finnish), and p.Tyr453Cys (European Caucasian) (17). Till date, more than 100 disease causing variants has been identified in the *ADA2* genes (**Supplementary Table**). Hotspot variants for bone marrow failure, vasculitis and PRCA were R169Q,

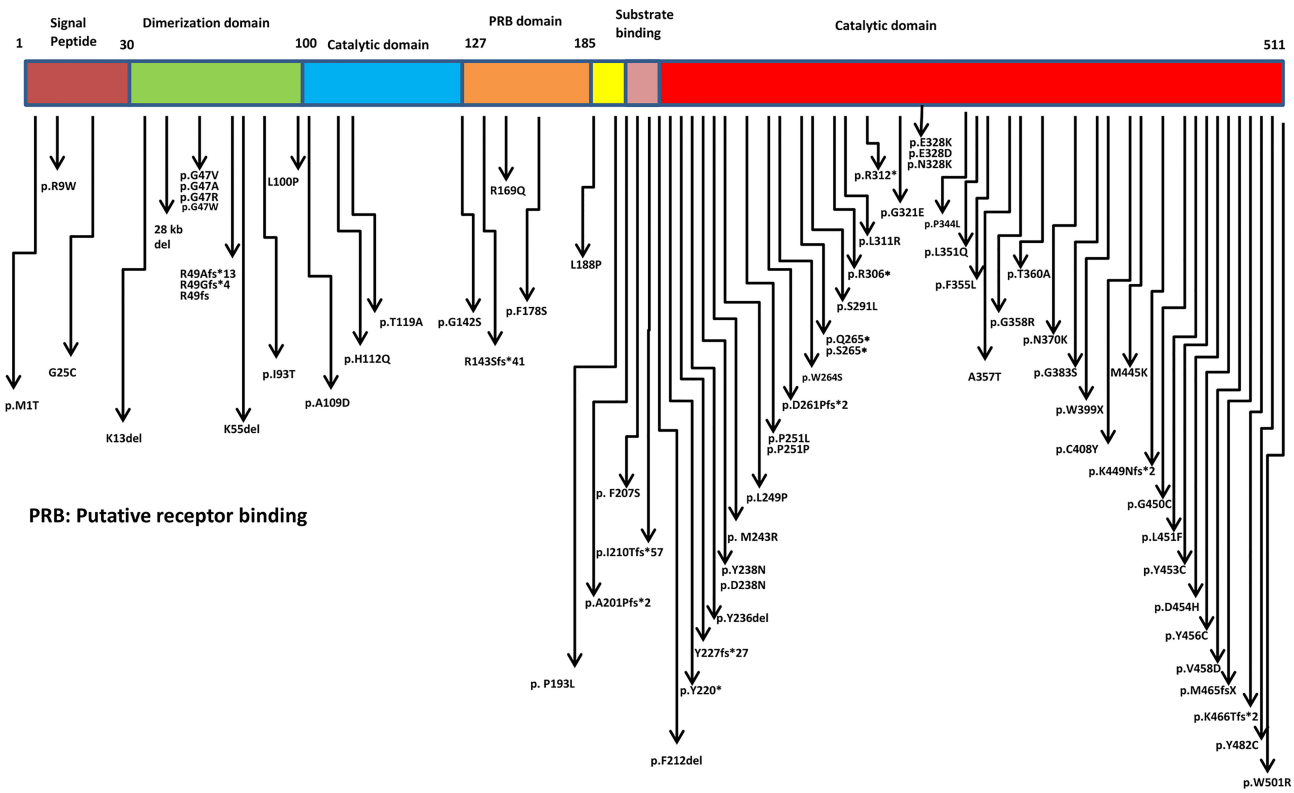

FIGURE 2 | Schematic representation of mutations in ADA2 protein.

G47R and G358R respectively. The most commonly seen variation was of missense type. Other than the hotspot variant some frequently observed variations in ADA2 were G25C, G47A, R49 Gfs*4, R49Afs*13, F178S, L188P, P251L, R306X, L351Q, T360A, Y453C, K466Tfs*2. Variants associated with haematological manifestations are enumerated in (**Table 1**). In general, variants leading to complete (or almost complete) loss of ADA2 enzyme activity have been associated with a predominant haematological phenotype (35).

7 OVERVIEW OF TREATMENT IN DADA2

Treatment of patients with DADA2 primarily depends on the clinical presentation. Besides specific therapy, supportive care (e.g. wound/ulcer care, antimicrobials for infections, etc.) is also essential to ensure better outcomes.

7.1 Anti-Inflammatory Therapy

Corticosteroids are widely used in acute phase of the disease; however, patients often show only a modest response. Disease flares are common while tapering steroids. A steroid-refractory course has also been described (2, 30, 43). TNF blockade is the therapy of choice for vasculitic and inflammatory manifestations. Etanercept and adalimumab are the commonly employed TNF inhibitors. In resource constrained settings, thalidomide may be used as an alternative due to its anti-TNF activity. Carosi et al. have reported the effectiveness of thalidomide in controlling disease activity in 7 patients (14). IL-6 blockers (tocilizumab) are also effective in controlling inflammation; however, recurrence of stroke has been noted (12, 23, 54, 63, 64). Tocilizumab has been used successfully in the patient with Castleman disease and DADA2. IL-1 blockade has not been noted to be of significant therapeutic benefit in patients with DADA2 (65).

7.2 Treatment of Immuno-Hematological Manifestations

In patients with hypogammaglobulinemia, immunoglobulin replacement therapy and antibiotic prophylaxis (21, 35) are the usual treatment modalities. Hematological manifestations are usually refractory to glucocorticoids (30). Other immunosuppressive drugs (azathioprine, mycophenolate mofetil, cyclosporine and anti-thymocyte globulin) have shown variable response in conditions like PRCA and other hematological phenotypes (35, 66). Rituximab has been shown to result in a favorable response in patients with autoimmune cytopenias (32). Mild manifestations (e.g. lymphopenia) may respond to TNF-blockers (66–68); however, TNF-blockade is ineffective for treatment of severe hematological manifestations (e.g. bone marrow failure and PRCA/DBA) (33, 35).

TABLE 1 | Variants associated with haematological manifestations.

S. no.	Amino acid change	cDNA position	Erythroblastopenia	Marrow dysfunction	AICs	Others
1	p.M1T	c.2T>C		+ F	+ ES	
2	p.G47R	c.139G>C				+ MAS/HLH
3	p.G47W	c.139G>T	+	+ N, F		
4	p.G47A	c.140G>C		+ P		
5	p.G47V	c.140G>T		+ N	+ HA	
6	p.R49Gfs*4	c.144delG	+			
7	p.R49Afs*13	c.143dup	+		+ HA	+ MAS/HLH
8	p.R49fs	c.144dupG	+	+ N		
9	p.I93T	c.278T>C			+ ES	
10	p.H112Q	c.336C>G	+	+ N, P		
11	p.R131Sfs*53	c.393del		+ P		
12	p.H133Lfs*44	c.396_397del			+ HA	
13	p.R169Q	c.506G>A	+	+ F, N, P	+ HA	+ MAS/HLH
14	p.F178S	c.533T>C	+	+ F		
15	p.T187P	c.559A>C			+ ES	
16	p.L188P	c.563T>C	+	+ N	+ HA, ES	
17	p.F207S	c.620T>C	+			
18	p.I210Tfs*57	c.629delT	+			
19	p.F212del	c.634_636delTTC		+ F		
20	p.Y220*	c.660C>A	+	+ P	+ HA	
21	p.Y227fs*27	c.680_681delAT	+			
22	p.E237R fs*30	c.709delC		+ N		
23	p.A247Qfs*16	c.714_738dup	+		+ HA	
24	p.V252Tfs*7	c.(753 + 168_754-229)del	+			
25	p.D261Pfs*2	c.781delinsCCATA		+ P		
26	p.S265*	c.794C>G		+ N		
27	p.R306X	c.916C>T	+			
28	p.L311R	c.932T>G	+	+ N, T		
29	p.R312*	c.934C>T		+ N		
30	p.G321E	c.962G>A	+	+ N, F		
31	p.Y353H	c.1057T>C		+ N, F		
32	p.G358R	c.1072G>A	+	+ N, F		
33	p.?	c.(1081 + 139_1082-92)del	+			
34	p.N370K	c.1110C>A	+	+ N		
35	p.W399X	c.1196G > A		+ N		
36	p.M445K	c.1334T>A	+			
37	p.K449Nfs*2	c.1346_1347insTT		+ F		
38	p.L451W	c.1352T>G	+			
39	p.L451F	c.1353G>T		+ N, F	+ HA	
40	p.Y453C	c.1358A>G		+ P, N		
41	p.D454H	c.1360G>C	+			
42	p.Y456C	c.1367A>G	+	+ N		
43	p.V458D	c.1373T>A		+ F		
44	p.M465fsX	c.1392dup	+	+ N		
45	p.K466Tfs*2	c.1397_1403delAGGCTGA	+	+ F		
46	p.Y482C	c.1445A > G	+			
47	p.(Ser483Profs*5)	c.1447_1451del		+ N	+ HA	
48	p.W501R	c.1501 T>C or T>A		+ F		
49		c.-47+2T>C	+			
50		c.1443-2T>A	+			
51		c.882-2A>G	+			
52		800-bp duplication	+			

AICs, Autoimmune cytopenias; F, Bone Marrow Failure; N, Neutropenia; P, Pancytopenia; ES, Evans Syndrome; HA, Hemolytic anaemia; HLH, Hemophagocytic lymphohistiocytosis; MAS, Macrophage Activation Syndrome; RCA, Red Cell Aplasia; DBA, Diamond-Blackfan anemia.
+Presence of manifestation.

7.3 Hematopoietic Stem Cell Transplantation (HSCT)

HSCT is the definitive treatment for hematological and immunological manifestations of DADA2. Hashem et al. have recently collated the multicentric experience of HSCT in 30 patients of DADA2 who underwent total 38 HSCTs. Indications for HSCT were bone marrow failure syndromes, autoimmune cytopenia, lymphoproliferation (benign or malignant) and immunodeficiency phenotypes. Overall survival after 2 years of follow-up was 97% and HSCT resolved the hematological phenotypes in all patients (33). Plasma ADA2 activity may be

restored to normal as early as 2 weeks post-transplant. HSCT may also benefit vasculopathic manifestations (33).

8 CONCLUSIONS

DADA2 may present with diverse hematological manifestations such as DBA/PRCA, immune cytopenia, bone marrow failure syndromes, lymphoproliferation and immunodeficiency. A detailed history, comprehensive clinical examination, and basic laboratory investigations are imperative in recognizing DADA2 in such scenarios. With increasing availability and decreasing costs of NGS, genetic testing seems to be a feasible option for diagnosing DADA2 (and other inborn errors of immunity) in patients with unexplained hematological manifestations.

AUTHOR CONTRIBUTIONS

RP: Inception of idea, writing of initial draft of manuscript, editing and critical revision of manuscript at all stages of its production, final approval of manuscript. AB, SSh, RK, VJ, and SL: writing of initial draft of manuscript, editing and revision of manuscript at all stages of its production, review of literature. MD, AJ, PV, and DS: Contributed to editing of manuscript, review of literature. AR and SSi: Critically revision of the manuscript at all stages of its production, final approval of manuscript, and review of literature. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.869570/full#supplementary-material

Supplementary Table 1 | Variants in *DADA2* gene.

Supplementary Figure 1 | *DADA2* gene sketch showing disease associated mutations in *ADA2* gene.

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Lymphoproliferation in Inborn Errors of Immunity: The Eye Does Not See What the Mind Does Not Know

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Inborn errors of immunity (IEIs) are a group of heterogeneous disorders characterized by a broad clinical spectrum of recurrent infections and immune dysregulation including autoimmunity and lymphoproliferation (LP). LP in the context of IEI may be the presenting feature of underlying immune disorder or may develop during the disease course. However, the correct diagnosis of LP in IEI as benign or malignant often poses a diagnostic dilemma due to the non-specific clinical features and overlapping morphological and immunophenotypic features which make it difficult to treat. There are morphological clues to LP associated with certain IEIs. A combination of ancillary techniques including EBV-associated markers, flow cytometry, and molecular assays may prove useful in establishing a correct diagnosis in an appropriate clinical setting. The present review attempts to provide comprehensive insight into benign and malignant LP, especially the pathogenesis, histological clues, diagnostic strategies, and treatment options in patients with IEIs.

Keywords: immunodeficiency, inborn errors of immunity (IEI), Ig/TCR gene rearrangements, lymphoproliferation, lymphoma

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INTRODUCTION

Lymphoproliferation (LP) in inborn errors of immunity (IEI) refers to persistent polyclonal, oligoclonal, or monoclonal proliferation of lymphoid cells in the clinical setting of primary immunodeficiency or immune dysregulation (1, 2). The incidence of LP in IEI varies from 0.7 to 18% (3). Typically, LP occurs during disease evolution in a patient with underlying primary immunodeficiency. However, it is difficult to assess the cases with LP as the presenting feature of IEI, posing a diagnostic challenge as there are no guidelines on the diagnosis and management of such cases. Moreover, the diagnostic and therapeutic approach toward cases with non-malignant LP in IEI is not clear. The current review will attempt to summarize the clinicopathological aspects and diagnostic approach to LP in IEI with the aim to provide an insight into early diagnosis and timely management of these cases.

CLINICAL ISSUES

Patients with LP associated with IEI usually present at a younger age with frequent extranodal involvement as compared to their immunocompetent counterparts. Clinical symptoms are non-specific and mimic those of an infection, inflammation, or neoplasia. The clinical features are characterized by chronic or recurrent lymphadenopathy, hepatosplenomegaly, extranodal infiltration, and/or peripheral blood lymphocytosis (4). In certain immune disorders such as autoimmune lymphoproliferative syndrome (ALPS) and X-linked lymphoproliferation (XLP), LP is the predominant feature at disease onset. In a diagnosed case of IEI, it is often challenging to accurately define LP as benign or malignant as both have different therapeutic and prognostic implications. Besides, the clinical suspicion and diagnosis of immune disorder underlying LP is a mammoth task and requires expertise. The relevance of correctly diagnosing and treating benign LP in IEI lies in the fact that it may not only act as a precursor lesion of lymphoid malignancy but also may be associated with an increased risk of hemophagocytic lymphohistiocytosis (HLH) (5). Thus, in a given case an apparently innocuous LP may have a sinister connotation. Treatment and prognosis of LP in IEI depend upon the severity of the underlying immune defect and need to be assessed individually.

DIAGNOSTIC ISSUES

A recent nomenclature has attempted to classify LP in the setting of immunodeficiency based on the morphology of lesion, viral infection status (EBV or HHV-8), and the clinical immunodeficiency state (2). With respect to LP in IEI, the morphological spectrum chiefly encompasses Epstein-Barr virus (EBV)-associated B-cell LPDs similar to the histological lesions observed in post-transplant lymphoproliferative disorders (PTLD). These include B-cell lymphoid hyperplasias: follicular, plasmacytic, and infectious mononucleosis (IM)-like; polymorphous B-cell LP; indolent lymphomas; aggressive non-Hodgkin lymphomas (NHL); and classical Hodgkin lymphoma (HL)- like LP (2). In general, T/NK-cell LPs are rare in IEI, the majority being benign/reactive CD8+T-cell infiltrates. Certain morphological clues in tissue biopsies may help to predict the underlying immune disorder [**Figure 1**]. In a recent review investigating benign and malignant LP, distinct histopathological alterations in the distribution of CD4+

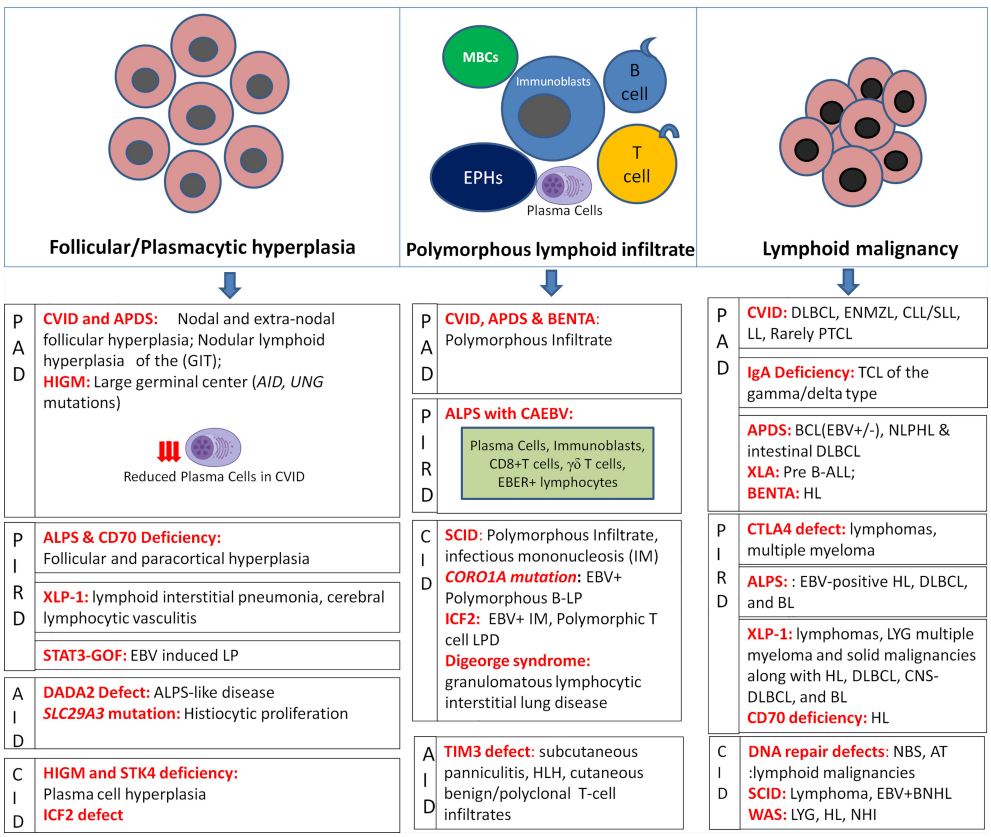

FIGURE 1 | Histopathological alterations in LP associated with IEIs depicting hyperplasias, polymorphous lymphoid infiltrates, and lymphoid malignancies as per IEI disease phenotypes (MBCs, monocytoid B-cells; EPHCs, epithelioid histiocytes).

follicular helper T-cells, follicular dendritic cells, and mantle zone naïve B-cells correlate with the different patterns in the development of germinal centers (6). Nevertheless, achieving a specific diagnosis is often challenging owing to the significant degree of morphological and immunophenotypic overlap between the benign and malignant lesions such as polymorphous B-cell LP is a masquerader of Hodgkin lymphoma (HL) and benign CD8+T-cell/histiocytic infiltrates in common variable immunodeficiency (CVID) could be easily confused with mimicking T- large granular lymphocytic leukemia (T-LGLL) leading to overtreatment.

PATHOPHYSIOLOGY AND HISTOPATHOLOGICAL ALTERATIONS

Combined Immunodeficiency Disorders

In severe combined immunodeficiency (SCID), loss of T/NK cell-mediated immune surveillance leads to uncontrolled EBV-driven B-cell LP that may progress into lymphoma (7). Homozygous and hypomorphic missense mutations in *CORO1A* may present as early-onset EBV+B-LP as a result of CD4+CD45RA+ T-cell lymphopenia and impaired invariant NKT-cell development and survival defects (8). Dedicator of cytokinesis-8 (DOCK8) deficiency/ hyper IgE syndrome (HIES) leads to impaired NK-cell function and increased predisposition to EBV+ lymphomas (9). In hyper IgM syndrome (HIGM) with *CD40LG/CD40* mutations, the defective antibody response to antigens results in the malignant transformation of B-cells (10). Serine/threonine-protein kinase 4 (STK4) deficiency, has been associated with nodal and extra-nodal EBV+ LP and B-cell lymphoma suggesting the role of EBV infection in inducing LP (11). Inducible tyrosine kinase (ITK) deficiency is associated with progressive CD4+T-cell and NKT-cell lymphopenia, and hypogammaglobulinemia resulting in EBV viremia and immune dysregulation leading to massive LP (12).

Histopathological changes in SCID comprise polymorphous LP characterized by systemic proliferation of highly polymorphous B-lymphoid cells showing plasmacytoid and immunoblastic differentiation that may also progress into HLH and EBV+B-NHL (1). EBV-encoded RNA (EBER)+ polymorphous CD20+ B-cell LP and DLBCL have been reported in patients with hypomorphic *CORO1A* mutations (8). DOCK8 deficiency has been reported to manifest as young-onset EBV+ and EBV- lymphomas and EBV+ pulmonary lymphomatoid granulomatosis (LYG) (9). In HIGM, the absence of germinal centers in lymph nodes and a massive extranodal accumulation of plasma cells that secrete IgM, particularly in GIT, to T-LGLL, HL, and EBV+B-NHL have been reported (1). STK4-deficiency is associated with plasma cell hyperplasia and EBV+ B-cell polymorphous LP, and B-cell lymphomas showing plasmacytic differentiation (11). ITK deficiency is chiefly associated with EBV+B-LPD, especially HL (12)

Combined Immunodeficiencies With Associated or Syndromic Features

Wiskott-Aldrich syndrome (WAS) patients have an increased predisposition to develop EBV-driven LYG, NHL, and HL usually following a long duration of non-malignant LP due to defective immune surveillance against the virus-infected cells (13). Patients with DNA repair defects, Nijmegen breakage syndrome (NBS), and ataxia-telangiectasia (AT) show a high predisposition to lymphoid malignancies at a young age as a result of genomic instability, chromosomal abnormalities, combined B- and T-cell immunodeficiency, and radiation hypersensitivity (14, 15). Histologically, WAS is associated with EBV+ extranodal clonal angioinvasive B-LP (LYG), HL (nodular sclerosing and lymphocyte depleted), and B-NHL, particularly DLBCL (1). Notably, T-NHL, T-acute lymphoblastic leukemia/ lymphoma (T-ALL/LBL), and clonal non-malignant T-cell proliferations are more common than B-NHLs in AT, and NBS (1). An increased propensity for T-prolymphocytic leukemia (T-PLL) has been reported in children with AT (1). Among B-cell lymphomas, classical HL, diffuse large B-cell lymphoma (DLBCL), and Burkitt lymphoma (BL) are commonly described (1). Immunodeficiency with centromeric instability and facial anomalies type 2 (ICF2) is a DNA-methylation disorder with a high susceptibility to EBV infection manifesting as EBV+ IM, HLH, chronic active EBV infection (CAEBV), HL, large B-cell lymphoma, and polymorphic non-clonal T-cell LPD (16). Patients with Di-George syndrome may present with CD8+ granulomatous cutaneous T-cell lymphoma and granulomatous lymphocytic interstitial lung disease (GLILD) characterized by ill-defined non-necrotizing lymphohistiocytic granulomas, CD20+ B-cell-rich lymphoid nodules, CD4+ T-cell-rich interstitial pneumonia, and peribronchiolar follicular hyperplasia with reduced regulatory T-cells (Tregs) (17). Homozygous Post-meiotic segregation 2 (*PMS2*) mutations, a DNA mismatch-repair defect is characterized chiefly by poor antibody responses and low B-cell number and is associated with B- and T-cell leukemia/ lymphoma besides colorectal cancer and brain tumors (18). The other CIDs associated with increased risk of lymphomas include Bloom syndrome, ligase 1 and MCM4 deficiencies, and cartilage hair hypoplasia (19).

Predominantly Antibody Deficiencies

In CVID, LP is one of the prominent clinical features accounting for approximately 20% of cases (20). Lymphadenopathy has been associated with the expansion of transitional B-cells (21). A recent report based on the United States Immunodeficiency Network (USIDNET) registry reported lymphoma in 8% of 1091 CVID patients (22). Lymphoma is the second major cause of mortality in CVID after chronic lung disease (23). B-cell lymphomas arise chiefly from germinal center-experienced mature B-cells that have undergone somatic hypermutation of Ig genes, correlating with a higher frequency of DLBCL, extranodal marginal zone lymphoma (ENMZL), and HL in CVID (24). Granulomatous CVID has been reported to involve every organ system occurring in 8-20% of CVID cases (25). The prevalence of LP in X-linked agammaglobulinemia (XLA) is extremely low (approximately 0.7%) in comparison to other IEIs (26). Nevertheless, the presence of lymphadenopathy in XLA is considered a matter of concern as it may harbor a lymphomatous process. Patients with selective IgA deficiency

are usually asymptomatic, although may rarely develop infections, autoimmune disorders, and malignancies (27, 28). Considering the shared genetic basis between CVID and selective IgA deficiency, a higher incidence of malignancies has been observed, primarily gastrointestinal (29, 30). Activated phosphoinositide 3-kinase delta syndrome (APDS) is chiefly characterized by recurrent respiratory tract infections, CAEBV, immune dysregulation, and recurrent or persistent LP presenting as lymphadenopathy, splenomegaly, mucosal nodular lymphoid hyperplasia, and lymphoma (31). CAEBV results from impaired NK and CD8+ T cell-mediated killing of infected cells by EBV. Persistent EBV infection and B-cell proliferation due to constitutive activation of B-cell intrinsic PI3Kδ signaling contribute to lymphomagenesis in APDS (32). B-cell expansion with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and T-cell anergy syndrome (BENTA) is a rare disorder featured by constitutive activation of the NF-κB signaling pathway leading to EBV-driven polyclonal B-cell LP manifesting as lymphocytosis, splenomegaly, and lymphadenopathy (33). NFKB1 deficiency is a clinically heterogeneous PAD with impaired function of B-cells with or without T-cell dysfunction and is characterized by recurrent infections, cytopenias, and EBV+ B-LP (34). Defects in activation-induced cytidine deaminase (*AID*) and uracil DNA glycosylase (*UNG*) in HIGM are frequently associated with lymphoid hyperplasia (35).

The morphological spectrum of LP in CVID is heterogeneous ranging from benign follicular hyperplasia and paracortical expansion with EBV-positive B-cells including large pleomorphic Reed-Sternberg (RS)-like cells to clonal but non-malignant B-cell nodular lymphoid hyperplasia of the gastrointestinal tract with a near-total absence of plasma cells to DLBCL, ENMZL, CLL/SLL, LPL, HL and rarely peripheral T-cell lymphoma (PTCL) (1). Notably, CD8+ cytotoxic T-cell proliferations are common in peripheral blood and hepatic sinusoids of CVID patients which is often difficult to differentiate from T-LGLL (36). Precursor B-ALL has been reported in XLA (37). Though rare, a few case reports have described T-cell lymphoma of the γδ type in selective IgA deficiency (28). APDS is frequently associated with non-malignant gastrointestinal and respiratory nodular mucosal lymphoid hyperplasia. These lesions show atypical follicular hyperplasia with disrupted and hyperplastic germinal centers, attenuated mantle zones, and perisinusoidal aggregates of monocytoid B-cells (31). There is hyperplasia of CD4+PD1+ follicular T-helper cells and PD1+CD57+CD8+ senescent T-cells and IgM+ plasma cells with reduced IgG+ plasma cells. EBV-associated oligoclonal polymorphous- B-cell LP has also been reported comprising polymorphous lymphoid infiltrate comprising of B- and T-cells, plasma cells, epithelioid histiocytes, and monocytoid B-cells. APDS shows an increased predisposition to EBV+/- B-cell lymphomas including intestinal DLBCL, and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) (32). A dual clinicopathological presentation of APDS with early-onset HLH followed by HL has also been reported (38). EBV+ polymorphous B-LPD and HL are described in BENTA disease (33). NFKB1-deficient patients may present with generalized lymphadenopathy with EBV+ reactive lymphoid hyperplasia of increasing clinical severity (34). *AID* and *UNG* mutated HIGM shows enlarged lymph nodes with large germinal centers (19, 35).

Diseases of Immune Dysregulation

In ALPS, homozygous mutations in *FAS* and *FASL* genes are associated with impaired cytotoxicity and severely reduced activation-induced cell death (AICD) in B- and T-cells resulting in clinically severe disease and massive LP including lymphomagenesis (39). Besides ALPS, *Caspase-8* mutations may also present as end-organ LP, granulomatous inflammation, mesenteric lymphadenopathy, and recurrent EBV infection (40). CD25, CD122, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) deficiencies, and signal transducer and activator of transcription 3 (*STAT-3*) gain-of-function mutations lead to impaired Treg function leading to impaired suppression of effector T cells causing immune dysregulation with autoimmunity and may present with LP with recurrent EBV infections (19, 41). The estimated risk of malignancies in affected patients with CTLA-4 deficiency is approximately 12.9% (41). CTLA4 insufficiency and biallelic *LRBA* mutations share similar clinical features and are also associated with granulomatous lymphocytic interstitial lung disease (GLILD) (17, 42). Elevated STAT3 signaling leads to defects in phosphorylation of STAT5 and STAT1, impaired Treg function, enhanced T-helper cell-17 differentiation, LP, and systemic autoimmunity (19, 42). XLP1 and 2 are associated with EBV-driven HLH and LP due to severely reduced iNKT cells, although XLP1 shows an increased risk of lymphomagenesis (19). Another defect in CTP synthase 1 (*CTPS1*) also leads to fatal viral (EBV) infections which results in LP and non-Hodgkin B cell lymphoma (19, 43). Similarly, RAS guanyl-releasing protein 1 (*RASGRP1)* deficiency has been involved with T-cell lymphopenia and EBV-associated B-cell lymphoma (44). Accelerated loss of TCR repertoire diversity has been observed in these cases. Additionally, CD70, CD137(4-1BB), CARMIL2, PRKCD, and CD27 deficiencies and X-linked magnesium EBV and neoplasia (XMEN) exhibit unique susceptibility to EBV infection and associated LP as a result of impaired T-cell activation and proliferation (19). Impaired CD27-CD70 interactions lead to loss of CD4+ and CD8+ T-cell response, impaired EBV killing, and CAEBV with hypogammaglobulinemia (45). Interestingly, CD70 deficiency may clinically mimic periodic fever syndrome (45). Fanconi anemia-associated protein 24 (FAAP24) deficiency, a familial HLH syndrome, results in fatal EBV-driven LP due to the failure of cytotoxic CD8+T cells to kill EBV-infected B-cells (19).

Histopathological features in ALPS include marked proliferation of non-clonal CD4-/CD8-CD45RA+ CD57 +TCRαβ+ cytotoxic (TIA+ and perforin+) double-negative T-cells (DNTs), involving peripheral blood, lymph nodes, liver, spleen, and extranodal sites, a few cases may show γδ-T-cell proliferation (36, 46). Follicular and paracortical hyperplasia and PTGC have been frequently described (1). HL, classical and

NLPHL, DLBCL, Rosai-Dorfman disease (RDD), and rarely PTCL have been well-described in ALPS (1). Histopathological features of LP in ALPS may overlap with CAEBV, especially in those cases where classic ALPS morphology is not evident. In such cases, florid paracortical hyperplasia comprising plasma cells, immunoblasts, EBER+ lymphocytes, cytotoxic T-cells, and γδ-T-cells is the predominant finding (46). CTLA-4 deficient patients have an increased risk of solid and lymphoid malignancies including lymphomas and multiple myeloma. The reported cases are mostly EBV-positive HL, DLBCL, and BL (41). XLP1 associated LP ranges from non-malignant LP including lymphoid interstitial pneumonia, LYG, cerebral lymphocytic vasculitis, HLH, and severe IM, to malignant lesions like HL, DLBCL, CNS DLBCL, and BL (47). Patients with 4-1BB deficiency have been diagnosed with EBER+ HL and CD20-CD38+ DLBCL on lymph node biopsies (48). CD70 deficiency can show varied presentations ranging from EBV +IM, reactive follicular and paracortical hyperplasia to HL (45). CD27 deficiency manifests as EBV+HLH and lymphomas (19).

Autoinflammatory Disorders

Patients with T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3) deficiency usually present with subcutaneous panniculitis, HLH, cutaneous benign/polyclonal T-cell infiltrates, and panniculitis-like T-cell lymphoma of subcutaneous tissue and mesenteric fat (49). Deficiency of adenosine deaminase 2 (DADA2) is a recently described autoinflammatory disorder that is caused by bi-allelic mutations in the *CECR1* gene encoding adenosine deaminase 2 (*ADA2*). Around 30% of cases present with splenomegaly and 10% present with lymphadenopathy. DADA2 may have varied clinical presentations ranging from vasculopathy, chronic liver disease, immune cytopenias, immunodeficiency, hypogammaglobulinemia, and LP (50). LP in DADA2 includes T-LGLL, ALPS-like disease, and HL. Germline mutation in the *SLC29A3* gene causes histiocytosis-lymphadenopathy plus syndrome which is characterized by abnormal histiocytic proliferation and accumulation in lymph nodes, liver, spleen, GIT, CNS, skin, and kidneys causing organ damage (19).

Phenocopies of PID

Somatic activating mutations in *NRAS* and *KRAS* genes may produce an ALPS-like phenotype, Ras-associated autoimmune leukoproliferative disorder (RALD) that is featured by chronic non-malignant LP, autoimmune cytopenias, monocytosis, and hypergammaglobulinemia. LP is usually indolent, although myeloid malignancies have been reported in a few cases (19, 51).

Laboratory Diagnosis

Morphological Assessment

Histopathological changes of LP often correlate with the underlying IEI. However, the pathologist should be aware of the potential diagnostic pitfalls while analyzing tissue biopsies, especially CD8+ cytotoxic T-cell infiltrates and polymorphous B-cell LP that could easily be mistaken for lymphoma leading to aggressive therapy. It is critical to understand that in IEIs, a major proportion of LP is constituted by polyclonal/oligoclonal, benign/reactive lymphoid infiltrates and every case is not a lymphoma, although the risk of malignant transformation is higher as compared to immunocompetent individuals. Detection of EBV encoded small RNA by *in-situ* hybridization (EBER-ISH), EBV encoded nucleic antigen (EBNA) and latent membrane protein (LMP) in lymphoid cells correlates well with EBV-infection and LP. EBV infection may cause downregulation of B-cell markers (CD20, CD79b, and CD19) and upregulation of CD30 (1). Immunohistochemistry (IHC), although used routinely for the detection of B-, T- cell, histiocytes, and plasma cell lineage antigens, should be interpreted with caution and in an appropriate clinical context. The tumor cells express high levels of programmed death-ligand 1 (PDL1) in CVID associated EBV+ DLBCL and HL (24). Immune checkpoint inhibitors are used to block this immune checkpoint protein, PDL1 expressed by tumor cells in HL to augment the T-cell mediated immune response. Although rare, it is difficult to diagnose T-cell lymphomas in IEIs other than DNA-repair defects owing to a high frequency of reactive/non-malignant T-cell LP. In such cases, a comprehensive approach including clinical phenotype, size of lymph node/lesion, architectural effacement, cellular/nuclear atypia, TCR gene rearrangements, chromosomal aberrations, and immunophenotyping should be followed (36).

Flow Cytometry

Flow cytometric *in situ* hybridization (Flow-FISH) is a powerful diagnostic tool for EBV+LP as it can quantify and phenotypically characterize the EBER-positive lymphocytes (52). In CVID, flow cytometry-based detection of an increased proportion of $CD21^{low}$ B-cells and transitional B-cells (CD38+IgM+) and decreased proportion of class-switched memory B-cells correlates with splenomegaly, granulomatous inflammation, and lymphadenopathy (53). Several fluorescent-labeled antibodies against B/T/NK-cell lineage markers are routinely used to differentiate between reactive versus neoplastic populations. Kappa/lambda ratio (>3 or <0.5) is a more reliable indicator of the clonal process, although the complete absence of light chain expression has been described (54). T-cell receptor-Vβ (TCR-Vβ) repertoire assay is used to assess clonality in suspected T-cell LP covering almost 70% of the TCR repertoire. It is recommended for peripheral blood samples only as it gives inconclusive results with tissue samples and bone marrow aspirates (54). Nevertheless, flow cytometry is an invaluable and robust test with an accuracy of 70-90% in LPDs (54) [**Supplementary Table 1**].

Molecular Assays

Molecular assays used to assess clonality in suspected LP include Southern blot hybridization (SBH), restriction fragment length polymorphism (RFLP), and polymerase chain reaction (PCR)-based assays like fragment length analysis using capillary electrophoresis (Gene scan) and next-generation sequencing (NGS). These assays are largely based on detecting clonal *Ig/TCR* gene rearrangements that are random recombination events between one of several *V*, (*D*), and *J* segments generating unique

exon sequences encoding antigen-bearing sites on Ig or TCR molecules for each lymphocyte. These events occur during the initial B- and T- cell development and impart diversity to Ig/TCR molecules (up to 10^{12}) expressed by each lymphocyte. When identical sequences are shared by cells, it represents the clonal nature of that population, and identification of such homogeneous/clonal or heterogeneous/polyclonal populations forms the basis of clonality testing in suspected LP. A standardized and optimized multiplex PCR has been developed that targets nearly all *Ig/TCR* (*IgH, IgK, TCRγ, TCRβ, and incomplete IgH D-J and TCRβ D-J*) rearrangements by fragment length analysis (55). Due to the remarkably increased rates of clonality detection in B- and T-cell malignancies, these multiplex PCR assays have become gold-standard for clonality testing in LP. The interpretation is based on the identification of specific patterns of peaks generated by Genescan classified as clonal, polyclonal, pseudoclonal, multiple products, and non-evaluable (55). Although applicable in more than 95% of cases for routine clonality diagnostics across multiple centers, these assays have their own pitfalls, particularly when low-intensity clonal signals are generated (55). Moreover, they do not provide information about the clone-specific sequence and need to be run in duplicate for reproducible results. With the advent of NGS technology, *Ig/TCR* rearrangements may be targeted with higher sensitivity and improved clonality detection rate. The advantages of NGS over other molecular assays include a) improved detection of clonal populations in clinical samples with lymphoid cells undergoing somatic hypermutation b) the patient-specific index clone could be accurately sequenced and monitored for clonal evolution c) the entire spectrum of gene rearrangements in a sample could be visualized simultaneously, and d) clonal association between different lesions may be determined (56). Amplicon-based and targeted NGS-based strategies have been established to detect rearrangements and translocations including *IGH, IGK, IGL, TRA, TRB, TRG, and TRD* genetic loci in clinical samples (57). The targeted NGS panels are more economical, with less sample requirement, and multiplexing in a single assay, and enable uniform reporting of results along with the potential to detect copy number variations (CNV), translocations, somatic mutations, and indels associated with LPDs, thus achieving a sensitivity of 99.6% (57).

Despite the availability and recent advancements in diagnostic techniques, many patients still remain undiagnosed and are either undertreated or overtreated. Being a complex disorder, it is important to understand the clinical phenotype and pathophysiology of LP in IEIs. We attempt to propose a diagnostic algorithm of investigating patients with LP with regard to specific IEIs (**Figure 2**).

Treatment

Improved survival with allogeneic reduced-intensity conditioning-hematopoietic stem cell transplant (RIC-HSCT) has been achieved in patients with IEI and B-cell lymphoma. These patients are treated with conventional chemotherapy and anti-CD20 monoclonal antibody (Rituximab) to achieve remission prior to the transplant. EBV infection in the pre-and post-transplant setting mandates initiation of therapy [Rituximab or EBV-cytotoxic T-lymphocytes (EBV-CTLs)] (58). HSCT is the definitive mode of therapy for many IEIs with LP including SCID and WAS. Apart from this, there are targeted therapies for some of the IEIs. Though management of CVID relies on long-term immunoglobulin replacement therapy (IVIg) and antibiotic prophylaxis, surveillance for complications is important (59). Follow-up for complications including malignancies, lymphoma, and non-neoplastic LP is important followed by appropriate therapy (22, 60).

Children with APDS show a significant reduction in infections on IVIg. HSCT with medium- or reduced-intensity conditioning has shown effective results (31, 61, 62) Autoimmune manifestations of APDS require immunosuppressive therapy (63). Rituximab has proven beneficial in the management of non-neoplastic LP. Inhibition of the downstream mTOR pathway by Sirolimus or Rapamycin decreases both non-neoplastic and neoplastic lymphoproliferation including regression of cutaneous T-cell lymphoma (31). In the European society for immunodeficiencies (ESID APDS) registry, a significant benefit (19/25) in the non-neoplastic LPD was seen on therapy with Sirolimus (64). Also, direct inhibition of the activated PI3Kδ by selective PI3Kδ inhibitors such as Leniolisib has shown a decrease in lymph node and spleen size in APDS (65).

In ALPS, treatment depends on manifestations and complications. Autoimmune manifestations, especially cytopenias require immunosuppressive therapy with corticosteroids and IVIg followed by steroid-sparing agents (66). The two most common first-line steroid-sparing agents used currently are mTOR inhibitor (Sirolimus) and Mycophenolate mofetil (MMF) (66–68). Long-term studies have shown durable improvement in lymphadenopathy and splenomegaly within 3 months of initiating sirolimus (69, 70). Patients with lymphadenopathy need to be closely monitored for lymphoma development. Severe and refractory cases require HSCT.

The definitive treatment of XLP is HSCT. However, therapy needs to be tailored according to the symptoms and infection profile. IVIg replacement therapy is essential in almost all patients to reduce infection risk and treat hypogammaglobulinemia. EBV+ patients need therapy with Rituximab. HLH needs aggressive therapy with high dose IVIg, Rituximab +/- HLH protocol. The presence of lymphoma needs surgery and chemoradiation with standard protocol followed by HSCT (71, 72).

In DADA2, management of vasculopathy and stroke is well-established with the use of anti-TNF therapy, however other manifestations including LP, cytopenias, and malignancy need immunosuppressive therapy. Treatment of these includes aggressive chemoradiation with IVIg therapy and TNF blockers to prevent disease progression on case to case basis (73). HSCT is the definitive therapy for the immunological, vascular, and hematological phenotype (74).

No clear treatment protocols for BENTA have been available as of now, however, patients receive multiple therapeutic modalities in the form of steroids, Rituximab, and Sirolimus with variable benefits. Long-term surveillance is important due to the increased risk of B-cell malignancy (75).

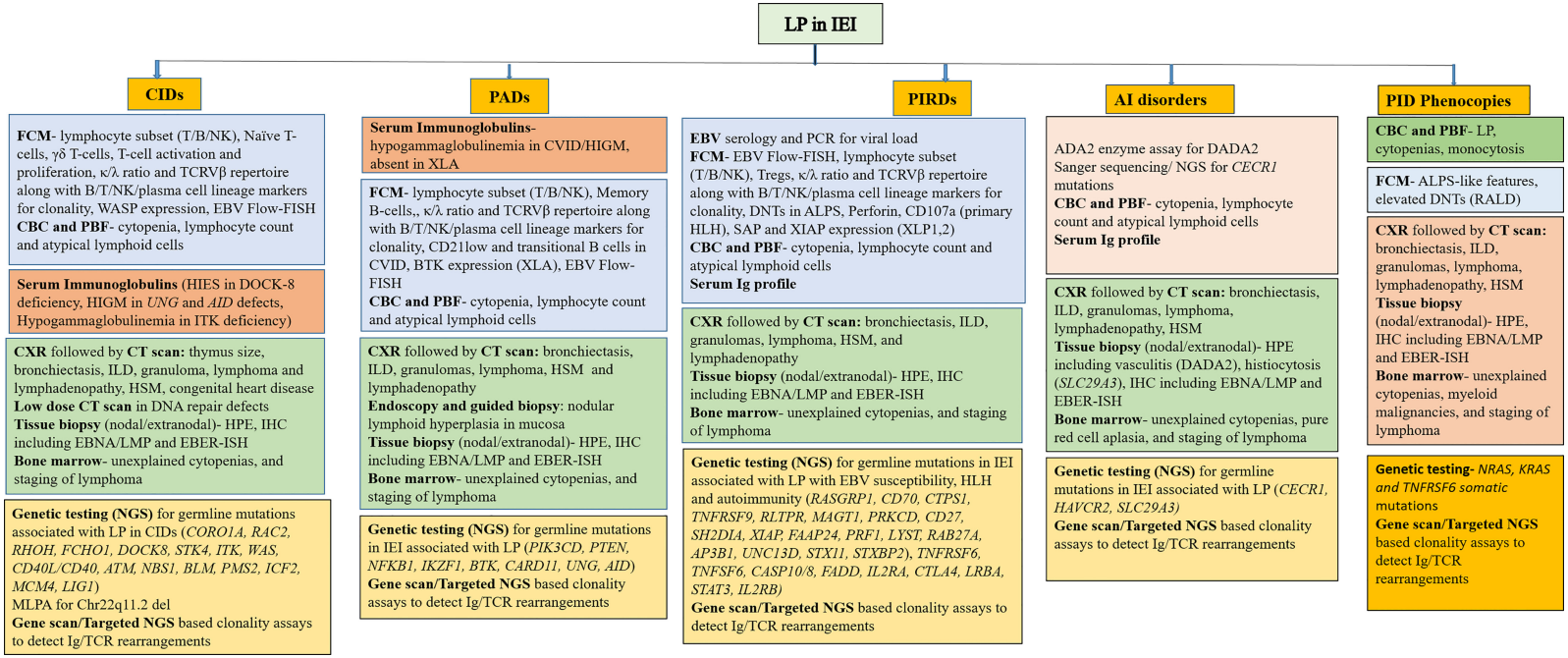

FIGURE 2 | Diagnostic algorithm of investigations for LP in IEI according to clinical phenotype (FCM, flow cytometry; CBC, complete blood count; PBF, peripheral blood film; CXR, chest X-ray; CT, computed tomography; HPE, histopathological examination; NGS, next-generation sequencing; PCR, polymerase chain reaction; IHC, immunohistochemistry; EBER-ISH, Epstein-Barr virus-encoded RNA- *in-situ* hybridization; MLPA, multiplex ligation-dependent probe amplification; FISH, fluorescence *in-situ* hybridization).

STAT3 gain-of-function mutations manifest with a variety of clinical symptoms including lymphoproliferation, and multiorgan autoimmunity. Patients have been treated with Azathioprine and MMF with poor response. Later, IL-6 blockers and HSCT were also tried (76, 77). Recently, the upstream inhibitors, such as Tocilizumab (anti-IL-6 receptor monoclonal antibody) and Janus kinase (JAK) inhibitors are available (78, 79).

CONCLUSIONS

In the patients with IEI, benign/reactive LP must be thoroughly investigated and monitored closely for malignant transformation. Any early-onset nodal or extranodal LP in an appropriate clinical context must be investigated for an underlying IEI. EBV testing must be done routinely in all patients. Flow cytometry and molecular genetics should be used in conjunction with morphology, especially in challenging cases.

AUTHOR CONTRIBUTIONS

SSh designed and supervised the manuscript. SSh, RP, GA, MS, KA, RT, MD, and PV reviewed the literature and wrote the manuscript. AR and SSi reviewed and edited the draft. All authors read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.856601/full#supplementary-material

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Underlying Inborn Errors of Immunity in Patients With Evans Syndrome and Multilineage Cytopenias: A Single-Centre Analysis

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Background: Evans syndrome (ES) is a rare disorder classically defined as the simultaneous or sequential presence of autoimmune haemolytic anaemia and immune thrombocytopenia, but it has also been described as the presence of at least two autoimmune cytopenias. Recent reports have shown that ES is often a manifestation of an underlying inborn error of immunity (IEI) that can benefit from specific treatments.

Aims: The aim of this study is to investigate the clinical and immunological characteristics and the underlying genetic background of a single-centre cohort of patients with ES.

Methods: Data were obtained from a retrospective chart review of patients with a diagnosis of ES followed in our centre. Genetic studies were performed with NGS analysis of 315 genes related to both haematological and immunological disorders, in particular IEI.

Results: Between 1985 and 2020, 40 patients (23 men, 17 women) with a median age at onset of 6 years (range 0–16) were studied. ES was concomitant and sequential in 18 (45%) and 22 (55%) patients, respectively. Nine of the 40 (8%) patients had a positive family history of autoimmunity. Other abnormal immunological features and signs of lymphoproliferation were present in 24/40 (60%) and 27/40 (67%) of cases, respectively. Seventeen out of 40 (42%) children fit the ALPS diagnostic criteria. The remaining 21 (42%) and 2 (5%) were classified as having an ALPS-like and an idiopathic disease, respectively. Eighteen patients (45%) were found to have an underlying genetic defect on genes *FAS*, *CASP10*, *TNFSF13B*,

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LRBA, *CTLA4*, *STAT3*, *IKBGK*, *CARD11*, *ADA2*, and *LIG4*. No significant differences were noted between patients with or without variant and between subjects with classical ES and the ones with other forms of multilineage cytopenias.

Conclusions: This study shows that nearly half of patients with ES have a genetic background being in most cases secondary to IEI, and therefore, a molecular evaluation should be offered to all patients.

Keywords: Evans syndrome, autoimmune cytopenias, inborn errors of immunity (IEI), immune dysregulation, autoimmune haemolytic anaemia (AIHA), ITP (idiopathic thrombocytopenic purpura), autoimmune neutropenia (AIN), ALPS (autoimmune lymphoproliferative syndrome)

INTRODUCTION

Evans syndrome (ES) is a rare disorder classically defined by the concomitant or sequential presence of autoimmune haemolytic anaemia (AIHA) and immune thrombocytopenia (ITP) (1–3), but it is also described as cytopenia due to the immune-mediated destruction of at least two blood cells lineages (4–6). It can be either idiopathic or secondary to other conditions, such as infections, inborn errors of immunity (IEI), autoimmune and rheumatologic diseases, malignancies, and drugs.

In paediatric age, IEI and in particular primary immunoregulatory disorders (PIRDs) play a relevant role in the development of autoimmune cytopenias (7–9). In the first reported paediatric cohort of ES, about 10% of patients were identified as having IEI (10). Since then, the increased use of techniques like next-generation sequencing (NGS) and whole exome sequence (WES) revealed closer relationships between ES and PIRDS as autoimmune lymphoproliferative syndrome (ALPS), ALPS-like disorders, and common variable immunodeficiency (CVID) that often present with autoimmune cytopenias (AC) (10–13), and outlined the role of variants on the clinical phenotype of ES. This was clearly shown by two recent studies. The first found seven pathogenic variants on *CTLA4*, *LRBA*, *STAT3*, and *KRAS* genes in a cohort of 18 children with ES (14), whereas the second identified an underlying genetic defect in 65% of cases and showed that patients carrying variants displayed a more severe disease and required more lines of treatment versus the ones without (15). Similar findings came from another multicentre study on 60 children where underlying immune dysregulation was detected in 42% of cases (6). This is relevant because the detection of specific monogenic defects may not only address a correct diagnosis, but also enable the use of targeted therapies (4, 16–18).

However, in the above mentioned large, multicentre studies, genetic analysis was not offered to all eligible patients but was performed according to the request of the attending physicians. In addition, financial access to molecular analysis may have somehow affected the prevalence and the type of underlying disorders (6, 14, 15). Moreover, the issue of the potential genetic and immunological differences between classical ES (association of AIHA and ITP) and other forms of multilineage cytopenia was not addressed.

The aim of this study is to evaluate the genetic background and the clinical/immunological features of a single-centre cohort of paediatric patients with ES and other multilineage cytopenias.

MATERIALS AND METHODS

Patients and Data

The clinical charts of all paediatric patients affected with classical ES and other multilineage cytopenias referred to our Unit between 1985 and 2020, identified *via* a clinical database, were reviewed.

AIHA was defined as the presence of haemolytic anaemia, a positive DAT, and the absence of other hereditary or acquired causes of haemolysis (16–18). AIN was defined as neutropenia due to the presence of indirect anti-neutrophil antibodies (19). ITP was defined as isolated thrombocytopenia (peripheral blood platelet count < $100{,}000 \times 10^9$/l) in the absence of other causes or disorders that may be associated (20, 21).

Patients presenting with the association of AIHA and ITP with or without autoimmune neutropenia (AIN) were defined as having a classical ES. Children suffering from AIHA and AIN or ITP and AIN were considered as having a multilineage autoimmune cytopenia (MAC). ALPS was defined according to the revised diagnostic criteria by Oliveira et al. (22) which needs the presence of two required criteria in addition to a primary or secondary accessory criterion to state a definitive and probable diagnosis, respectively. Patients with both definitive and probable diagnoses were considered in the ALPS group of our cohort. Patients who did not completely fulfil the ALPS diagnosis but, in addition to cytopenia, presented with at least one required or primary additional criterion of ALPS diagnostic criteria were classified as having an ALPS-like disorder. Patients without diagnostic criteria of ALPS, ALPS-like, or any other underlying systemic disorder were considered as having a primary disease. Lymphoproliferation was defined as the presence of chronic (>6 months), non-malignant, non-infectious lymphadenopathy, hepatomegaly, or splenomegaly. Other immune abnormalities were defined as the presence of any of the following: inflammatory bowel diseases, autoimmune hepatitis, autoimmune thyroiditis, celiac disease, and auto-antibody positivity (ANA, ENA, ASMA, ASCA, ANCA, anti-ADAMTS13, anti-parietal, anti-dsDNA, anti-SSA/Ro, or LAC).

Data on demographics, clinical features, laboratory and immunological findings, management, and outcome were collected.

All adult subjects provided written informed consent to participate to this study, while parental consent was obtained for children, as approved by the Istituto Gaslini Ethical Committee.

The study and all analyses conformed to the 1975 Declaration of Helsinki. Novel variants reported here for the first time have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and assigned accession number SCV001424053-SCV001424130.

Lymphocyte Immunophenotype and Serum Biomarkers

Peripheral lymphocyte subsets were evaluated from whole blood using an eight-colour immunostaining panel (lyse and wash procedure), a FACSCanto II flow cytometer (BD, Franklin Lakes, NJ, USA) equipped with three lasers (blue, red, violet), FACSDiva™ software (BD), and a large panel of RUO monoclonal antibodies and fluorochromes variously combined (all BD). CD3+CD4-CD8-TCRαβ+ T cells (double-negative T cells, DNTs) were calculated on total lymphocytes. An *in vitro* FAS-induced apoptosis test was considered pathological when positive in two separate assays.

Genetic Analysis

DNA was isolated from peripheral blood samples of patients and their parents, when available, and tested for a selected list of genes through an NGS-based gene panel already reported (23). Sample library preparation and sequencing, and successive bioinformatics analyses, including variant annotation and interpretation, were carried out as described in Grossi et al. (23). The effect of variants was classified according with American College of Medical Genetics ACMG criteria (24) which are implemented in the VarSome database (www.varsome.com). In particular, the family segregation, population frequencies, functional prediction, and zygosity were taken into consideration.

Relevant variants were confirmed by polymerase chain reaction (PCR) amplification and direct Sanger sequencing of the corresponding DNA segments. X-inactivation analysis was performed using peripheral blood genomic DNA undigested or digested with restriction endonucleases sensitive to cytosine methylation (HpaII). PCR was carried out using two primers flanking the STR in the HUMARA gene. The PCR products were run on ABI PRISM 3130 (Applied Biosystems, Foster City, CA, USA). The ratio of active/inactive X chromosome was determined as described by Bolduc et al. (25).

Statistical Analysis

Continuous variables were described as the median (range), and categorical variables were described as number (percentage). Quantitative variables were analysed using the χ^2 test or Fisher exact test for small quantities. Differences between variables of various groups were considered significant when the P value was ≤ 0.05.

RESULTS

Forty patients (23 men, 17 women) with a median age at onset of 6 years (range 0–16) were studied. Twenty-two (55%) and 18/40 (45%) presented with ES and MAC, respectively. Seventeen out of 40 (43%) children met the ALPS diagnostic criteria. The remaining 18 (45%), 3 (7%), and 2 (5%) were classified as having an ALPS-like phenotype, CVID, and idiopathic cytopenia, respectively. Nine of the 40 (8%) patients had a positive family history of autoimmunity. Other immune abnormalities and signs of lymphoproliferation were present in 24/40 (60%) and 27/40 (67%) of cases, respectively. All patients but one required second- or further-line treatments which included mycophenolate mofetil (MMF) and sirolimus or both in 27 (67%), 18 (45%), and 14 (35%) cases, respectively.

The classical ES and MAC groups did not differ for any of the tested variables including family history of autoimmunity ALPS and ALPS-like/CVID phenotype with the exception of other immune abnormalities that were significantly more frequent (P = 0.03) in patients with MAC over those with classical ES. A trend of females to prevail in classical ES vs. MAC, without reaching statistical significance, was also observed **(Table 1).**

Genetic analysis was performed in all 40 patients, and variants were found in 18 (45%) of them. Variants were pathogenic/likely pathogenic in 13 and of unknown significance in 5 subjects. Patient carrying variants were not differently distributed in the classical ES and MAC groups. All variants defined as pathogenic were previously described or functionally validated (26, 27). Of note, they were all related to PIRDs and IEI. In particular, in 5/18 (28%) patients the gene was involved in the pathogenesis of ALPS (4 *FAS*, 1*CASP10*) and in 7/18 (39%) subject variants were implicated in ALPS-like disorders (4 *TNFSF13B*, 1 *LRBA*, 1 *CTLA4*, 1 *STAT3*). The remaining 6/18 cases (33%) were found to have an impairment in genes related to other IEI. Overall, genetic variants were found in 10/18 (55%) of patients with ALPS. **Tables 2**, **3** show the clinical/immunological details and the molecular results of patients carrying genetic variants, and the characteristics of the remaining cases are reported in **Table 4**.

In order to see whether there were differences between the patients carrying variants versus those who did not, we divided the whole cohort in these two groups. We did not find any significant difference for any of the tested variables (**Table 5**). However, although not reaching statistical significance, a greater need of second-line therapies was observed in the patients with variants (14/18, 78%) compared with the ones without (14/22, 64%). In line with this, patients carrying variants were also the only ones who required further treatment with stem cell transplantation (SCT): five of them were transplanted from haploidentical (3), sibling (1), and unrelated (1) donor, respectively, and are all alive and well at a median of 4.6 years after the procedure. Two patients (5%) died due to complication of the diseases. The median follow-up was 6.9 years (0,3–35).

DISCUSSION

Paediatric Evans syndrome is a very rare and challenging disease. To the best of our knowledge, this study reports on the largest monocentric cohort of children, homogenously screened with molecular analysis, and confirms the presence of underlying disorders in a considerable number of cases, highlighting the important role of genetic assessment in this setting of patients.

TABLE 1 | Clinical and immunological differences according to type of cytopenia.

	ES n 22 (%)	MAC n 18 (%)	p
Presence of genetic variant	11/22 (50)	7/18 (39)	0.48
Females	12/22 (55)	5/18 (27)	0.08
Familiar history of autoimmunity	7/22 (32)	2/18 (11)	0.11
Associated immunological features[a]	10/22 (45)	14/18 (78)	0.03*
ALPS phenotype at onset[b]	10/22 (45)	7/18 (39)	0.67
ALPS-like/CVID phenotype at onset	10/22 (45)	11/18 (61)	0.32
Alive	20/22 (91)	18/18 (100)	0.18
DNT[c]> 1.5%	15/22 (68)	15/18 (83)	0.27
Need for second-line therapy	12/22 (55)	8/18 (44)	0.52
Sequential cytopenia	12/22 (55)	10/18 (56)	0.94

[a]*Celiac disease, presence of autoantibodies including antinuclear, anti-neutrophils, anti-neutrophil cytoplasmic, anti-smooth muscle, anti-thyreoperoxydase, anti-thyroglobulin, lupus anticoagulant, anti-ADAMTS13, extractable nuclear antigen, cold agglutinins.*

[b]*Presence of required and accessory diagnostic criteria for ALPS.*

[c]*Double-negative T cells (CD3+ CD4- CD8- TCR$\alpha\beta$+).*

**Statistically significant.*

This holds especially true in the case of IEI that are known to have overlapping phenotypes due to the incomplete penetrance of genetic defects and other still unknown epigenetic factors (9, 11).

Previous multicentric studies have already shown the presence of underlying immune dysregulation in most ES patients. However, in these studies, differently from ours in which all patients were genetically tested, patients underwent genetic analysis based on a prescription of the attending physician, on the availability of the diagnostic tools over the years, on financial access to analysis, and on the severity of the disease (6, 14, 15). This might have generated a selected cohort of patients that were more likely to show an underlying defect and might explain the lower detection rate in our patients (48%) compared to that of the larger multicentric French cohort (68%) (15). In addition, our results were obtained using NGS gene panel analysis, thus implying that a deeper investigation with WES or WGS (whole genome sequencing) might have increased the detection rate. Such further analysis should be taken into consideration in patients with negative NGS results, since the presence of other immuno-pathological manifestations in the majority of our patients is in keeping with the idea that both ES and MAC represent an epiphenomenon of some still unknown IEI.

As expected, most of the variants found in our cohort were involved in genes causing CVID, ALPS, or ALPS-like syndromes. Interestingly, patients carrying *CTLA4* (27) and *DADA2* (26) variants initially presented with an exclusive haematological phenotype which was followed by more typical signs of their diseases during adolescence/adulthood, highlighting that, in the paediatric age, cytopenia can be the first sign of a more complex disease which can show its complete phenotype later in life. In this respect, the *DADA2* patient's peripheral cytopenia worsened due to the occurrence of severe bone marrow failure. The coexistence of immune-mediated destruction of blood cells and marrow failure was also shown in both patients (Pt 9 and Pt 16) carrying two very rare variants of unknown significance on the *CARD11* gene, described to be damaging in most scores and also included in the GUK (guanylate kinase-like) domain, critical for protein's function, where other pathogenic variants have been reported. The phenomenon of the immune-mediated interplay between bone marrow and peripheral blood in the pathogenesis of cytopenia in IEI has already been highlighted by our group (30) and has clinical implications since these patients deserve a particular alert and specific follow-up.

The pathogenic variant *IKBKG* was found in a female presenting with isolated ES during childhood and showing further signs of immune dysregulation during adolescence. The abnormal chromosome X inactivation documented in our female patients carrying a X-linked disorder—known to be the cause of incontinentia pigmenti, ectodermal dysplasia, and immunodeficiency—may explain the milder phenotype. In addition, also the different degree of the protein impairment (NF-kappa-B essential modulator—NEMO), which may depend on the type of variant, may have contributed to the milder clinical issues (31, 32).

As expected, most patients showed higher levels of DNTs, which are well known to be raised not only in patients with ALPS but also in association with ALPS-like or CVID phenotypes (33). This indicates that increased DNTs may represent an important initial screening tool for patients with ES and MAC that can address subsequent specific immunological investigations (4, 34–36).

No clinical and immunological differences were noted comparing patients with or without a genetic diagnosis. Similarly, apart from a slight—although not significant—female predominance in patients with ES and a statistically significant higher presence of immune abnormalities in MAC cases, we did not notice other differences between both groups. This strengthens the concept that multilineage cytopenia can be an epiphenomenon of an underlying disorder, regardless of the involved cell line. Nonetheless, the several additional manifestations of autoimmunity and of immune dysregulation noted in patients with MAC may reflect the more heterogeneous genetic background we found in this group.

As already reported in the French cohort (15), most patients needed second- or further-line immunosuppressive therapies which, in most cases, were successful. In fact, treatments as mycophenolate mofetil and sirolimus, well known to be effective in autoimmune cytopenias (37, 38), represent an appropriate

TABLE 2 | Clinical/immunological characteristics of patients carrying pathogenic/likely pathogenic variants related t o IEI (abnormal results in bold).

Pt, sex	Type of cytopenia	Clinical phenotype	Age (years) at diagnosis	Other clinical signs	Other abnormalities	Lymphocyte subpopulations					ALPS Cytokines			Immunoglobulin levels			Variant°	GnomADMAF	Varsome	SCT	Status
						L tot (/mmc)	T (%)	B (%)	NK (%)	DNT (%)	Il10 (pg/mL)	IL18 (pg/mL)	Vit B12 (ng/L)	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)					
1, F	ITP, AIHA	ALPS	7	LPR	No	1,900	83	**6**	9	**2**	Na	Na	**1,581**	**Na**	**17**	81	*FAS* p.Glu256Gln	\	LP	No	Alive
2, M	ITP, AIHA	ALPS	6	LPR	No	1,757	78	15	**16.4**	**5.8**	**35**	**550**	921	**2,000**	100	43	*FAS* p.Glu 245Lys	\	P	No	Alive
3, F	ITP, AIHA	ALPS	1	LPR	No	**970**	75	**9.4**	**13.3**	**13**	**40**	**950**	**10,233**	1716	120	93	*FAS* p.Gln273His	\	P	No	Alive
4, F	ITP, AIHA	ALPS	12	LPR arthritis,	Anti-parietal ab	**510**	65	19	**13.5**	**2.2**	7	425	370	**600**	97	66	*CTLA4* p.Cys583fs*13	\	LP	No	Alive
5, F	ITP, AIHA, AIN	ALPS	2	LPR arthritis, sclerosing cholangitis	Anti TPO, anti-Tg, CD, ANA, LAC	**670**	87	**5.3**	2	**4.7**	0,2	265	999	**3,556**	199	244	*IKBKG** p.Glu125Lys	0.00186	LP	No	Alive
6, F	ITP, AIHA, AIN	CVID	2	LPR	AI hepatitis, ANCA	4,125	62	20	**12.6**	**2.4**	0	**2,100**	0	1,185	119	14	*CARD11* p.Arg967Cys	0.0000294	VUS	No	Alive
7, F	ITP, AIHA, AIN	ALPS	5	LPR	No	5,060	97	**2.6**	1	**5.1**	14	**5,250**	374	**254**	**13**	299	*ADA2* p.Thr187Pro/ p.Leu188Pro	/;0.00000882;/	LP/LP/LP	No	Dead
8, M	ITP, AIHA	Idiopathic	3	No	Recurrent infections	**315**	85	**0.2**	**14**	**1.7**	**385**	268	268	695	136	61	*STAT3* p.Lys658Arg (mosaicisms) *LIG4* p.Arg278His HOMO	\	P	Yes	Alive
9, F	ITP, AIN	ALPS	1	LPR recurrent fevers	DAT; CD, ASMA, AI hepatitis	5,890	82	13	3.7	**2.6**	Na	Na	Na	955	54	67	*LRBA* Arg655Ter HOMO	0.0000147	P	No	Alive
10, F	ITP, AIN	ALPS	11	LPR recurrent fevers	No	1,690	73	19.9	1.1	**6**	**79**	**1,175**	**2,000**	1,689	293	110	*FAS* p.Cys179Arg	\	P	No	Alive
11, M	ITP, AIN	ALPS	9	LPR	CD	**60**	61	28	0	**3.5**	4	550	Na	Na	Na	Na	*STAT3^* p.Arg152Trp	\	LP	No	Alive
12, M	ITP, AIN	ALPS-like	2	No	AI hepatitis, ENA	**730**	**45.8**	38.2	**8.1**	**3**	Na	Na	**1,100**	**1,815**	26	**20**	*CARD11* p.Val1009Ile	0.000559	VUS	Yes	Alive
13, M	AIHA, AIN	ALPS-like	2	LPR, MAS	Cold agglutinins	**749**	**48**	15	4.1	0.4	Na	Na	400	**549**	**19**	**17**	*RAG1* p.Arg507Gln HOMO	\	LP	Yes	Alive

*IEI, inborn errors of immunity; AI hepatitis, autoimmune hepatitis; AIHA, autoimmune haemolytic anaemia; AIN, autoimmune neutropenia; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASMA, anti-smooth-muscle antibodies; B, benign; BMF, bone marrow failure; CD, celiac disease; DAT, direct antiglobulin test; DNT, double negative T cells; ENA, extractable nuclear antigen antibodies; ES, Evans syndrome; SCT stem cell transplant; ITP, immune thrombocytopenia; LAC, lupus anti-coagulant; LP, likely pathogenic; MAC, multilineage autoimmune cytopenia; MAS, macrophage activation syndrome; Na, not available; P, pathogenic; Tg, thyroglobulin; TPO, thyroid peroxidase; VUS, variant of unknown significance; LPR, lymphoproliferation (Chronic, > 6 months, non-malignant, non-infectious lymphadenopathy or splenomegaly or both). °Variant zygosity is always meant as heterozygosity unless differently reported * A ratio of active/inactive × chromosome equal to 70:30, demonstrating that a moderate skewing was detected. This finding suggests a correlation between skewed × inactivation and phenotype in carriers of X-linked disease. ^ Gain of function (28).*

TABLE 3 | Clinical/immunological characteristics of patients carrying pathogenic or of unknown significance variants related to risk factors for immune-dysregulation (abnormal results in bold).

Pt, sex	Type of cytopenia	Clinical phenotype	Age (years) at diagnosis	Other clinical signs	Other abnormalities	Lymphocyte subpopulations					ALPS cytokines			Immunoglobulin levels			Variant	GnomAD MAF	Varsome	SCT	Status
						L tot (/mmc)	T (%)	B (%)	NK (%)	DNT (%)	Il 10 (pg/mL)	IL18 (pg/mL)	Vit B12 (ng/L)	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)					
1, M	ITP, AIHA	ALPS-like	4	No	No	2,298	**53**	**44**	0.6	0.4	Na	Na	732	1,197	52	106	*TNFRSF13B* p. Ser194Ter	\	P	No	Alive
2, F	ITP, AIHA, AIN	CVID	3	LPR, arthritis, recurrent fevers	CD, ANA	1,129	72	11	**15**	5	0,9	**1,225**	807	**250**	**15**	101	*TNFRSF13B* p.Arg202His	0,000729	VUS	No	Alive
3, M	ITP, AIHA	ALPS-like	1	No	No	4,375	57	**31**	**9.5**	**2.7**	11,2	**800**	1,005	**1,785**	84	157	*TNFRSF13B* p.Gln57His	0,00019	VUS	No	Alive
4, F	ITP, AIN	CVID	1	Congenital malformations hydrocephalous BMF	No	**310**	**52**	35	0.5	0.8	Na	Na	Na	Na	Na	Na	*TNFRSF13B* p.Arg202His	0,000729	VUS	Yes	Alive
5, F	AIHA, AIN	ALPS	1	LPR	DAT, ASMA	3,045	74	**9**	1.6	**4.1**	**90**	**5,001**	**1,492**	**1,596**	119	69	*CASP10* p.Val410Ile	0,0419	B°	Yes	Alive

AIHA, autoimmune haemolytic anaemia; AIN, autoimmune neutropenia; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASMA, anti-smooth-muscle antibodies; B, benign; BMF, bone marrow failure; CD, celiac disease; DAT, direct antiglobulin test; DNT, double-negative T cells; ENA, extractable nuclear antigen antibodies; ES, Evans syndrome; SCT stem cell transplant; ITP, immune thrombocytopenia; LAC, lupus anticoagulant; LP, likely pathogenic; MAC, multilineage autoimmune cytopenia; MAS, macrophage activation syndrome; Na, not available; P, pathogenic; Tg, thyroglobulin; TPO, thyroid peroxidase; VUS, variant of unknown significance; LPR, lymphoproliferation (chronic, > 6 months, non-malignant, non-infectious lymphadenopathy or splenomegaly or both). °Abnormal functional test (29).

TABLE 4 | Clinical/immunological characteristics of patients without genetic variant (abnormal results in bold).

Pt, sex	Type of cytopenia	Clinical phenotype	Age (years) at diagnosis	Other clinical signs	Other abnormal immunological features	Lymphocyte subpopulations					ALPS cytokines			Immunoglobulin levels			Status
						L tot (/mmc)	T (%)	B (%)	NK (%)	DNT (%)	IL 10 (pg/mL)	IL18 (pg/mL)	Vit B12 (ng/L)	IgG (mg/dL)	IgA (mg/dL)	IgM (mg/dL)	
1, M	ITP, AIHA, AIN	ALPS	1,5	LPR	ANA	**690**	69	20	10.1	**15**	0	**550**	684	1,616	91	212	Alive
2, F	ITP, AIHA	ALPS-LIKE	6,8	LPR	No	**490**	87	**6**	**5**	1	0.9	**925**	378	834	105	50	Dead
3, F	ITP, AIHA	ALPS-LIKE	11,6	TTP	ANA	2,215	63	16	18	1	0	0	0	904	176	101	Alive
4, F	ITP, AIHA	ALPS-LIKE	0,3	No	No	1,785	81	**0.2**	**6**	**3**	1.3	280	361	839	13	4	Alive
5, M	ITP, AIHA	/	16,6	No	No	2,900	88	**3**	**0**	0.3	0	0	0	1,275	301	400	Alive
6, F	ITP, AIHA, AIN	ALPS-LIKE	5,3	LPR	ANA, ASMA	4,190	87	**1**	11	0.6	0	0	0	1,213	519	232	Alive
7, M	ITP, AIHA	ALPS-LIKE	6,8	No	ANA, LAC, ANCA, ENA, anti-dsDNA ab	**750**	67	17	14	3	13.2	375	0	546	62	25	Alive
8, M	ITP, AIHA	ALPS	11,6	LPR	Erythema nodosum	**1,050**	76	16	**0**	2	3	**2,000**	0	467	47	17	Alive
9, F	ITP, AIHA	ALPS	10,3	LPR	ANA, ENA, anti-dsDNA, anti-C3d, LAC, anti-Ro/SSA	1,270	84	10	**5**	3	0	**705**	283	1,412	163	107	Alive
10, M	ITP, AIN AIHA	ALPS-LIKE	15,3	No	ANA	1,580	83	**7**	10	3	0	0	0	1,229	220	67	Alive
11, F	ITP, AIHA	ALPS-LIKE	0,3	LPR	No	Na	Na	Na	Na	3	0	0	927	Na	Na	Na	Alive
12, M	ITP, AIN	ALPS-LIKE	8,8	No	Anti-N ab, anti-TG, anti TPO, DAT	1,400	66	12	19	**2.4**	0.9	200	669	921	107	47	Alive
13, M	ITP, AIN	ALPS	1,2	LPR	No	1,300	79	**4**	16	**2**	3.5	**2,150**	430	1,248	630	68	Alive
14, M	ITP, AIN	ALPS	14,9	LPR BMF	ANA, ENA, ASMA, CD	**850**	64	16	19	**2**	**305**	360	725	1,534	376	62	Alive
15, M	ITP, AIN	ALPS LIKE	10	LPR arthralgia	No	1,500	78	19	**2**	**2**	1.3	**640**	0	625	167	42	Alive
16, M	ITP, AIN	ALPS	11,2	LPR	DAT	**780**	69	18	12	**4**	3	**660**	356	820	33	87	Alive
17, M	ITP, AIN	ALPS LIKE	2,2	LPR	DAT	2,600	58	29	11	0	13	**600**	641	694	21	54	Alive
18, M	ITP, AIN	ALPS LIKE	14,8	LPR, recurrent fevers	ENA	**770**	83	**8**	**7**	**4.6**	2	**600**	274	**2,506**	228	174	Alive
19, M	ITP, AIN	ALPS LIKE	14,2	No	No	1,580	80	17	**1.5**	1.3	3	300	484	898	214	158	Alive
20, M	ITP, AIN	ALPS LIKE	7,6	LPR	DAT	1,890	82	12	**3.6**	**2.6**	Na	Na	**1,694**	1,457	36	143	Alive
21, M	AIHA, AIN	ALPS LIKE	6	LPR recurrent fevers, arthralgia, rash	ASMA, ASCA	1,190	73	12	13	**3.4**	Na	275	Na	**2,416**	286	236	Alive
22, F	AIHA, AIN	ALPS	12,6	No	ANA, anti-C3d, anti-dsDNA, SLE, Hashimoto's thyroiditis	**600**	85	**6**	**7**	**3.9**	3.2	421	655	1,075	205	45	Alive

anti-dsDNA ab, anti-double-stranded DNA antibodies; anti-N ab, anti-neutrophil antibodies; AIHA, autoimmune haemolytic anaemia; AIN, autoimmune neutropenia; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASCA, anti-Saccharomyces cerevisiae antibodies; ASMA, anti-smooth muscle antibodies; B, benign; BMF, bone marrow failure; CD, celiac disease; DAT, direct antiglobulin test; DNT, double-negative T cells; ENA, extractable nuclear antigen antibodies; ITP, immune thrombocytopenia; LAC, lupus anti-coagulant; LPR, lymphoproliferation (chronic, >6 months, non-malignant, non-infectious lymphadenopathy or splenomegaly or both); MAS, macrophage activation syndrome; Na, not available; SLE, systemic lupus erythematosus; Tg, thyroglobulin; TPO, thyroid peroxidase; TTP, thrombotic thrombocytopenic purpura.

TABLE 5 | Clinical and immunological differences according to variants.

	Tot (%)	Patients with variants (18/40, 45%)	Patients without variants (22/40, 55%)	p
Females	17/40 (43)	10/18 (56%)	7/22 (32%)	0.13
Familiar history of autoimmunity	9/40 (23)	5/18 (28%)	4/22 (18%)	0.47
Associated immunological features[a]	24/40 (60)	9/18 (50%)	15/22 (68%)	0.22
ALPS phenotype at onset[b]	17/40 (43)	10/18(56%)	7/22 (32%)	0.13
DNT[c] >1.5%	30/40 (75)	15/18 (83%)	15/22 (68%)	0.27
Signs of lymphoproliferation[d]	27/40 (68)	13/18 (72%)	14/22 (64%)	0.56
Need for second-line therapy	28/40 (70)	14/18 (78%)	14/22 (64%)	0.33
Sequential cytopenia	22/40 (55)	10/18 (56%)	12/22 (55%)	0.94

[a]*Celiac disease, presence of autoantibodies including antinuclear, anti-neutrophils, anti-neutrophil cytoplasmic, anti-smooth muscle, anti-thyroperoxydase, anti-thyeroglobulin, lupus anticoagulant, anti-ADAMTS13, extractable nuclear antigen, cold agglutinins.*
[b]*Presence of required and accessory diagnostic criteria for ALPS.*
[c]*Double-negative T cells (CD3+ CD4- CD8- TCR$\alpha\beta$+).*
[d]*Chronic (> 6 months), non-malignant, non-infectious lymphadenopathy or splenomegaly or both.*

approach for patients non-responding to steroids. Nonetheless, the identification of specific molecular defects, following a proper genetic screening, may lead to the administration of targeted therapies, as in the case of one patient of our cohort, affected with *CTLA4* haploinsufficiency, who was successfully treated with abatacept (27). Few non-responding patients—all with a demonstrated underlying defect—successfully underwent SCT (39).

In conclusion, both ES and MAC should be considered an epiphenomenon of underlying IEI which are detected in about half of patients. Therefore, in these cases, genetic screening has to be considered a fundamental step of the diagnostic work-up that should be offered to all patients who may potentially benefit from specific follow-up and treatments.

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 as follows: https://www.ncbi.nlm.nih.gov/clinvar/, SCV001424053-SCV001424130.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato etico Regione Liguria. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MM and DG designed the research and wrote the paper. AG, MaL, MT, and IC performed the genetic analysis. PT, EC, and MiL performed the laboratory assays and functional studies. EP, FF, GO, and AB contributed the clinical data. CD coordinated the research and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.869033/full#supplementary-material

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Autoimmune Cytopenias Post Hematopoietic Stem Cell Transplantation in Pediatric Patients With Osteopetrosis and Other Nonmalignant Diseases

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Autoimmune cytopenia (AIC) is a rare complication post hematopoietic stem cell transplantation (HSCT), with a higher incidence in nonmalignant diseases. The etiology of post-HSCT AIC is poorly understood, and in many cases, the cytopenia is prolonged and refractory to treatment. Diagnosis of post-HSCT AIC may be challenging, and there is no consensus for a standard of care. In this retrospective study, we summarize our experience over the past five years with post-HSCT AIC in pediatric patients with osteopetrosis and other nonmalignant diseases. All pediatric patients who underwent HSCT for nonmalignant diseases at Hadassah Medical Center over the past five years were screened for post-HSCT AIC, and data were collected from the patient's medical records. From January 2017 through December 2021, 140 pediatric patients underwent HSCT for osteopetrosis (n=40), and a variety of other nonmalignant diseases. Thirteen patients (9.3%) presented with post-HSCT AIC. Of these, 7 had osteopetrosis (17.5%), and 6 had other underlying nonmalignant diseases. Factors associated with developing AIC included unrelated or non-sibling family donors (n=10), mixed chimerism (n=6), and chronic GvHD (n=5). Treatment modalities included steroids, IVIG, rituximab, bortezomib, daratumumab, eltrombopag, plasmapheresis, and repeated HSCT. Response to treatment was variable; Seven patients (54%) recovered completely, and three patients (23%) recovered partially, still suffering from mild-moderate thrombocytopenia. Three patients died (23%), two following progressive lung disease and one from sepsis and multi-organ failure after a 3rd HSCT. In our experience, post-HSCT AICs in pediatric patients with nonmalignant diseases may pose a challenging post-transplant complication with a variable presentation and a wide spectrum of severity. A relatively high prevalence is seen in patients with osteopetrosis, possibly due to difficult engraftment and high rates of mixed chimerism. There is a dire need for novel treatment modalities for better management of the more severe and refractory cases.

Keywords: autoimmune cytopenia, hematopoietic stem cell transplantation, osteopetrosis, nonmalignant, immune thrombocytopenia, autoimmune hemolytic anemia, autoimmune neutropenia, pediatrics

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for various pediatric nonmalignant diseases including bone marrow failures, hemoglobinopathies, immune deficiencies, and inborn errors of metabolism (1). With constant improvement in supportive care and the introduction of novel treatments, outcomes of these transplants are improving and Indications for HSCT in pediatric nonmalignant diseases are constantly expanding (2).

Autoimmune cytopenia (AIC) post-HSCT is a relatively rare complication, with reported incidence ranging between 2.5-5% in pediatric patients. However, the incidence is much higher in patients undergoing HSCT for nonmalignant diseases, reaching 20-35% (3). The etiology of post-HSCT AIC is poorly understood and involves immune dysregulation and imbalance between autoreactive and autoregulatory lymphocytes during the process of post-HSCT immune reconstitution (4, 5). The differential diagnosis of post-HSCT AIC is broad, including viral infections, graft versus host disease (GvHD) related cytopenia, and drug toxicity causing myelosuppression. In many cases, the course of these complications is prolonged and often they are refractory to treatment, with high rates of morbidity and mortality. Therefore, diagnosis and management of post-HSCT AIC are often challenging, and there is no well-established standard of care (3–9).

In this study, we summarize our experience over the past five years with post-HSCT AIC in pediatric patients with nonmalignant diseases.

METHODS

Patients

In this retrospective study, all pediatric patients who underwent HSCT for nonmalignant diseases at Hadassah-Hebrew University Medical Center between January 2017 and December 2021 were screened for post-HSCT AICs. Criteria for inclusion in the AIC cohort included an AIC in any one of the three hematopoietic cell lines post-HSCT. Diagnosis of AIHA was established by a positive direct Coombs test and serum markers of hemolysis including elevated reticulocyte counts, low serum haptoglobin, and elevated serum LDH. Diagnosis of immune thrombocytopenia and autoimmune neutropenia was established by the exclusion of other causes for the cytopenia post HSCT such as drug toxicity, GvHD, or an underlying viral infection. Bone marrow biopsy was done in relevant cases to assess marrow cellularity and rule out cytopenia due to marrow insufficiency.

Data Collection

Data were collected from the patient's medical charts and included demographic, clinical, and transplant-related data. Clinical data included the underlying disease and indication for HSCT, time of appearance of AIC, type of AIC, treatment modalities, response to treatment, other post-HSCT complications, and outcomes. Transplant-related data included graft source and degree of match, conditioning regimen, GvHD prophylaxis, chimerism status at the time of AIC onset as per STR test, GvHD sites, severity, and management if applicable. "Full-donor" was defined as an STR test of 100% donor cells in the recipients' blood, "mixed chimerism" was defined as any percentage of donor cells in the recipients' blood below 100% and above 0%, and "recipient" chimerism was defined as 0% donor cells. This study was approved by our institutional Helsinki review board.

Statistical Analysis

Categorical variables were summarized as number and percentage, and continuous variables as median and range. Clinical and transplant characteristics were compared between patients who developed AIC and those who did not. Fisher's exact test was used to determine the relationship between categorical independent variables and the development of AIC. Comparison of continuous variables between patients with and without AIC was done by a T-test. A p-value of <0.05 was considered statistically significant. The statistical analysis was done using the software R version 4.1.2.

RESULTS

From January 2017 through December 2021, 140 pediatric patients underwent HSCT for a variety of nonmalignant diseases. Of these, thirteen patients (9.3%) presented with post-HSCT AIC. The clinical and transplant characteristics of screened patients, with a comparison between those who developed AIC and those who did not, are depicted in **Table 1**. No statistically significant differences were found between the AIC and the no-AIC groups in the clinical and transplant-related variables that were compared. The average age of the patients in the AIC cohort at the time of HSCT was 6.6 years, ranging from 5 months to 17.8 years. **Table 2** depicts the clinical and transplant characteristics of the 13 patients who were included in the AIC cohort.

Underlying Disease

Osteopetrosis is the most common nonmalignant underlying disease in our cohort. Out of all 140 patients who were screened for AIC, 40 had osteopetrosis, and out of the 13 patients who developed AIC, 7 (54%) had osteopetrosis. The prevalence of post-HSCT AIC in osteopetrosis patients in our cohort is 17.5%. Other underlying diseases in the AIC cohort include bone marrow failures (n=3), immune deficiencies (n=2), and Glanzmann thrombasthenia (n=1). Of note, in two patients (patients #5,11), the AIC developed following a second transplant.

Donor and Graft Source

In the AIC cohort, only 3 of the 13 patients were transplanted from HLA-matched sibling donors. The other 10 patients were transplanted from alternative donors: one from a one-allele

TABLE 1 | Clinical and transplant-related characteristics of all the pediatric patients with nonmalignant diseases who underwent HSCT during the study period at Hadassah Medical Center, with a comparison between the AIC and no-AIC groups.

		AIC	No AIC	All patients	P-value
Total		13 (9.3%)	127 (90.7%)	140	
Gender	(males, females)	9, 4	76, 51	85, 55	0.5673
Age at HSCT	(years)	6.6 (0.4-17.8)	5.2 (0.1-17.8)	5.4 (0.1-17.8)	0.4782
Disease	Osteopetrosis	7 (54%)	33 (26%)	40 (29%)	0.125
	Bone marrow failures	3 (23%)	33 (26%)	36 (26%)	
	Immune deficiencies	2 (15%)	49 (39%)	51 (36%)	
	Metabolic diseases	0	11 (9%)	11 (8%)	
	Other	1 (8%)	1 (0.8%)	2 (1.4%)	
Donor	Matched sibling	3 (23%)	45 (35%)	48 (34%)	0.2387
	Other matched family	2 (15%)	8 (63%)	10 (7%)	
	Haploidentical	0	7 (6%)	7 (5%)	
	Matched unrelated	3 (23%)	42 (33%)	45 (32%)	
	Mismatched unrelated (9/10)	5 (38%)	25 (20%)	30 (21%)	
Graft source	Bone marrow	9 (69%)	103 (81%)	112 (80%)	0.3972
	Peripheral blood	4 (31%)	22 (17%)	26 (19%)	
	Cord blood	0	2 (1.6%)	2 (1.4%)	
Chimerism	Full donor	7 (54%)	93 (73%)	100 (71%)	0.2665
	Mixed chimera	6 (46%)	30 (24%)	36 (26%)	
	Recipient		2 (1.6%)	2 (1.4%)	
GvHD	none	7 (54%)	76 (60%)	83 (59%)	0.1042
	grade I/II	4 (31%)	14 (11%)	18 (13%)	
	grade III/IV	2 (15%)	34 (27%)	36 (26%)	
2nd transplant		2 (15%)	10 (8%)	12 (8.6%)	0.3082
Mortality		3 (23%)	14 (11%)	17 (12%)	0.22

TABLE 2 | AIC cohort - patient and transplant characteristics.

Pt #	Primary disease	Age atTransplant(years)	Donor	HLA match	Graftsource	Conditioningregimen	GvHDprophylaxis
1	Osteopetrosis	7.3	grandmother	10/10	BM	Flu Treo TT ATG	CSA + MMF
2	Osteopetrosis	0.4	father	9/10	BM	Flu Treo TT ATG	CSA + MMF
3	Glanzmann Thrombasthenia	13.8	brother	10/10	BM	Rtx Cmp Bu Flu	CSA + MMF
4	VPS45 deficiency	0.8	unrelated	9/10	PBSC	Bu Flu TT ATG	CSA + MMF
5	Dyskeratosis Congenita	4.4	unrelated	10/10	BM	Cmp Flu Mel	CSA + MMF
			different unrelated	10/10	PBSC	Flu Treo ATG	CSA + MMF
6	Osteopetrosis	5.1	unrelated	9/10	BM	Flu Treo TT ATG	CSA + MMF
7	Osteopetrosis	1.4	unrelated	10/10	BM	Flu Treo TT ATG	CSA + MMF
8	Osteopetrosis	0.8	unrelated	9/10	BM	Flu Treo TT ATG	CSA + MMF
9	XLP1	14.9	unrelated	10/10	BM	Bu Flu ATG	CSA + MTX
10	Severe aplastic anemia	17.8	sister	10/10	BM	Flu Cy ATG	CSA + MMF
11	Severe aplastic anemia	16.4	sister	10/10	BM	Flu Cy ATG	CSA + MMF
			same sister	10/10	PBSC	Cmp Flu Cy TBI	CSA + MTX
12	Osteopetrosis	1.3	unrelated	9/10	PBSC	Flu Treo TT ATG	CSA + MTX
13	Osteopetrosis	1.1	unrelated	9/10	BM	Flu Treo TT ATG	CSA + MMF

HLA, human leukocyte antigen; GvHD, graft-versus-host disease; BM; bone marrow; PBSC, peripheral blood stem cells; Flu, fludarabine;Treo, treosulfan; TT ,thiotepa; ATG, anti-thymocyte globulins; Rtx, rituximab; Cmp, alemtuzumab (campath); Bu, busulfan;Cy, cyclophosphamide; TBI, total body irradiation; CSA, cyclosporin; MMF, mycophenolate mofetil; MTX, methotrexate;XLP1, x-linked lymphoproliferative disease 1.

mismatched father, one from his HLA-matched grandmother, 3 from matched unrelated donors, and 5 from one-allele mismatched unrelated donors. The graft source was bone marrow in 9 patients and peripheral blood stem cells (PBSC) in the other 4 patients.

Conditioning Regimen

The conditioning regimen was myeloablative in eleven of the thirteen patients of the AIC cohort; three of the patients received a busulfan-based regimen and eight patients received a reduced-toxicity, treosulfan-based regimen. Two patients, who were transplanted for severe aplastic anemia from matched siblings, received a reduced-intensity regimen with fludarabine and cyclophosphamide (120 mg/kg). One of them (patient #11) was a second transplant following secondary graft failure, and to enhance engraftment, he received a PBSC-collected graft, alemtuzumab for more intensive pre-HSCT lymphodepletion, and low-dose total body irradiation (3 Gy). All patients received serotherapy (either ATG or alemtuzumab), and cyclosporine with either mycophenolate mofetil or methotrexate for GvHD prevention.

AIC Characteristics

The AIC characteristics, various treatments, and outcomes are summarized in **Table 3**. The median time from HSCT to AIC onset was 74 days, ranging from the time of engraftment to one-year post-HSCT. In patients #5 and #11, the days to AIC onset were counted starting from their 2nd HSCT. Types of cytopenia were variable and included: isolated thrombocytopenia (n=3), isolated hemolytic anemia (n=1), bi-cytopenia (n=4), and tri-lineage pan-cytopenia (n=5).

Chimerism and GvHD

Six of the 13 patients had mixed chimerism and 7 patients had full-donor chimerism on STR tests during the time of AIC. Five of the patients had GvHD at the time of AIC, in four of them mild skin and GI GvHD, and in one patient (patient #9) extensive chronic GvHD of the GI tract, skin, and soft tissues. Another patient (patient #2) had severe GI GvHD which had already resolved at the time of AIC.

Treatments and Outcomes

In most cases, the first treatment line was steroids (n=10). Other treatment modalities included IVIG (n=3), rituximab (n=7), bortezomib (n=2), daratumumab (n= 4), eltrombopag (n=3), plasmapheresis (n=1), and repeated HSCT (n=2).

Seven patients (54%) completely recovered, three patients (23%) recovered partially and still have mild-moderate thrombocytopenia not requiring platelet transfusions, and three patients (23%) died. Of the deceased patients, one patient (patient #5) died three weeks after a 3rd HSCT due to sepsis and multi-organ failure, and two patients (patients #10,11) died from progressive lung disease concurrent with AIC.

DISCUSSION

Incidence Rates

Post-HSCT AICs may pose a challenging post-transplant complication (3). In this study we present our experience over the past 5 years with treating post-HSCT AIC in a cohort of 13 pediatric patients with nonmalignant diseases. These patients were identified and selected out of a total of 140 pediatric patients who underwent HSCT for a variety of nonmalignant diseases, with an incidence rate of 9.3% for developing post-HSCT AIC. This incidence rate in nonmalignant diseases compares well with recent literature. In a study from the Netherlands by Kruizinga et al. 26 patients with AIC were identified out of 531 post-HSCT pediatric patients, 22 of which had nonmalignant diseases, showing an incidence rate of 9.5% for AIC in nonmalignant diseases (5). In another study from California by Neely et al. 20 patients with AIC were identified out of 442 pediatric patients, out of which 9 had primary immune deficiencies (6% incidence), and 4 had other nonmalignant diseases (5.2% incidence) (6). A recent study by Lum et al. showed a cumulative incidence of 9.4% for developing post-HSCT AIC in patients with primary immunodeficiencies (9). And finally, a recent study by Galvin et al. described a cohort of 50 patients with post-HSCT AIC identified out of a cohort of 271 pediatric patients with nonmalignant diseases, showing a cumulative incidence of 18% (8).

The relatively high incidence rate of post-HSCT AIC observed in patients with nonmalignant diseases may be attributed to the chemotherapy-naive and relatively intact pre-HSCT hematopoietic system in these patients, which may increase the risk for persistence of auto-antibody secreting host plasma cells. Also, a more aggressive anti-GvHD approach in nonmalignant patients, including more pre-HSCT serotherapy and increased immunosuppressive treatments, may delay and skew the post-HSCT immune reconstitution process and increase the risk for developing AIC in these patients (10).

Malignant Infantile Osteopetrosis

Osteopetrosis is a group of rare genetic bone disorders characterized by excessive bone growth due to reduced osteoclast bone resorption (11). The severe, infantile, autosomal recessive disease, often named malignant infantile osteopetrosis (MIOP), is in most cases treatable by HSCT (12). These patients often come to transplant with severely damaged bone marrow due to overgrowing bone into the bone marrow niches and HSCT for these patients is often challenging. Due to high consanguinity rates in some populations in our region and the founder effect, many of our nonmalignant patients have MIOP and our center has gained vast experience with HSCT for treating this condition (13). Out of the 140 patients that were screened in this study, 40 patients had MIOP, of which 7 developed post-HSCT AIC (incidence of 17.5%). The relatively high incidence of AIC in these patients may be attributed to the difficult engraftment and high incidence of mixed chimerism in these transplants. The conditioning regimen we give for MIOP patients includes treosulfan, fludarabine, thiotepa, and ATG. This reduced-toxicity regimen may be less myeloablative than busulfan-based regimens, with relatively high rates of post-HSCT mixed chimerism in osteopetrosis patients (around 35% in our experience), yet highly immunosuppressive, with a significant impact on post-HSCT immune reconstitution (13). This combination of partial engraftment and intense immune suppression may set the stage for developing AIC in these patients.

Associated Factors

Ten of the thirteen patients in our cohort were transplanted from donors which were either unrelated or non-sibling family donors (**Table 2**). Of the three patients who were transplanted from matched-sibling donors, one was a patient with Glanzmann Thrombasthenia, who was treated with multiple blood products pre-HSCT and thus came to transplant with a hyper-sensitized immune system, and another was a patient with severe aplastic anemia, post 2nd PBSC-collected HSCT from his matched sister, following graft failure.

The high prevalence of alternative, non-matched sibling donors in our cohort compares well with other studies that have defined patients who were transplanted from unrelated

TABLE 3 | AICs, treatments and outcomes.

Pt #	Time from HSCT to AIC initial symptoms (days)	Medications at time of initial AIC symptoms	AIHA	ITP	AIN	Duration of cytopenia	Treatments for AIC (duration/doses)	Response to treatment	Chimerism in STR test during AIC	GvHD	Outcome
1	55	CSA	√	√	√	10 days	steroids (4 weeks), IVIG (2 doses)	CR	95-98%	no	alive and well
2	356	none	√	√		ongoing	steroids (2.5 months), eltrombopag (1 year), rituximab (3 doses), 2nd HSCT	CR	56-26%	severe GI	alive and well
3	since HSCT	CSA		√		ongoing	rituximab (4 doses), eltrombopag (3.5 years)	PR	84%-full	no	mild-moderate thrombocytopenia
4	103	CSA	√	√		7 months	steroids (8.5 months), rapamycin (2 months), rituximab (7 doses), abatacept (4 doses), bortezomib (5 doses), daratumumab (6 doses)	CR	full donor	no	alive and well
5	77	CSA, prednisone	√	√	√	20 months	steroids (6 months), rapamycin (3 months), rituximab (4 doses), bortezomib (5 doses), daratumumab (4 doses), 3rd HSCT	No R	full donor	mild skin and GI	deceased
6	103	none	√			2 months	steroids (2.5 months), IVIG (1 dose), rituximab (4 doses), daratumumab (5 doses), plasma-pheresis (2 times)	CR	14-33%	no	alive and well
7	74	none	√	√	√	1 month	no treatments	CR	full-> 45% -> full donor	mild skin and GI	alive and well
8	39	CSA		√	√	ongoing	steroids (4 weeks), rituximab (4 doses), daratumumab (6 doses), eltrombopag (6 months)	PR	full donor	no	moderate thrombocytopenia
9	280	Jakavi		√		1 month	steroids (2 weeks), IVIG (3 doses)	CR	full donor	chronic skin, eyes, GI	chronic GvHD
10	since HSCT	CSA	√	√	√	3 months	steroids (4 weeks)	No R	full donor	no	deceased
11	since HSCT	CSA	√	√	√	5 months	steroids (2 months)	No R	full donor	mild GI	deceased
12	99	CSA	√	√		1.5 months	steroids (5 weeks)	CR	24-52%	mild skin	alive and well
13	since HSCT	CSA		√		ongoing	steroids (3 weeks), rituximab (4 doses)	PR	full donor	no	moderate thrombocytopenia

AIC, autoimmune cytopenia; HSCT, hematopoietic stem cell transplantation; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia; AIN, autoimmune neutropenia; STR, short tandem repeats; GvHD, graft-versus-host disease; CSA, cyclosporin; IVIG, intra-venous immunoglobulins; MSC, mesenchymal stem cells; GI, gastrointestinal; EBV, Epstein-Barr virus; PTLD, post-transplant lymphoproliferative disease. CR, Complete response; defined cases where complete resolution of the cytopenia was observed. PR, partial response; defined cases where partial improvement in cytopenia was achieved, without a need for blood-product support. No R, no response; defined cases refractory to treatment in which patients continued to require blood-product support. Since HSCT = cytopenias which presented prior to engraftment and persisted as AIC.
"√", indicates involvement of the specific cytopenia in the clinical presentation of the patient.

donors as a risk group for developing AIC. Additionally, six of the patients in our cohort (46%) were mixed chimeras at the time of AIC onset, suggesting a role of residual antibody-secreting host cells in the pathogenesis, and the lack of myeloablation as a risk factor in the development of AIC (3–8).

Five of the patients (38%) in our cohort had manifestations of GvHD during the AIC. While GvHD is an alloimmune phenomenon involving donor immune recognition of host cells, AIC is thought to be an autoimmune process, involving donor immune recognition of donor-origin hematopoietic cells. However, the pathophysiology in both of these post-HSCT immune complications shares similar elements, both involving impaired immune reconstitution and immune dysregulation. In both, the impaired peripheral tolerance may be attributed to a lack of functional T regulatory (Treg) cells (3). Therefore, an association between GvHD and AIC is plausible.

Unfortunately, due to our relatively small sample size, we have failed to demonstrate statistically significant associations between these factors and post-HSCT AIC.

Treatment Options

Corticosteroids are usually the first line of treatment for AIC and almost all patients in our cohort were initially treated with either intra-venous methyl-prednisone or oral prednisolone. IVIG, also frequently given as initial treatment, was given to only three patients in our cohort (**Table 3**). In non-responders to first-line treatment, targeting antibody-secreting B-cells with rituximab is usually the next step. Although the response rate to rituximab reaches 60-80% in the literature (3), in our cohort all steroid non-responders received a trial of rituximab, and none of them responded to this treatment. Newer treatment modalities targeting plasma cells, such as bortezomib and daratumumab, are gaining popularity in the treatment of multiple myeloma and have recently shown efficacy in treating AIC. Bortezomib, a 26S proteasome inhibitor, decreases the production of IgG antibodies by eliminating plasma cells and has been reported to effectively treat post-HSCT AIHA (14, 15). In our cohort, two patients received bortezomib, with no response. Daratumumab, an anti-CD38 monoclonal antibody developed to target malignant

plasma cells in multiple myeloma, was also reported to effectively treat refractory post-HSCT AIC in several recent case reports (16). In our cohort, four patients received daratumumab, and in one of the patients (patient #4), a dramatic recovery from a refractory AIHA following the failure of several other treatments was observed (17). However, in three other patients in our cohort, there was no response to daratumumab, suggesting multifactorial pathogenesis of this complication and refractoriness to treatment in some cases.

Thrombopoietin receptor agonists such as eltrombopag and romiplostim, are used for the treatment of persistent/chronic ITP and have been used for post-HSCT thrombocytopenia in recent years with promising outcomes (18). Three of our patients were treated with eltrombopag, and in two of them, there was a good response and improvement in platelet counts following treatment.

Other third-line treatment options we have tried in refractory cases included rapamycin, an mTOR inhibitor that has shown efficacy in refractory cytopenias (19), and abatacept, a fusion protein that inhibits T-cell activation by binding to CD80/CD86 on antigen-presenting cells thus blocking the CD28 interaction with T-cells (20). Both have failed to show significant responses in our patients. Plasmapheresis, done for direct removal of circulating antibodies, was tried in one refractory case (patient #6), who eventually responded to multiple treatments and completely recovered.

As a last resort, a second transplant may reset the whole immune system, aiming to restart the process of immune reconstitution, at the price of significant treatment-related morbidity and mortality. In our cohort, two patients ended up undergoing a repeat HSCT due to prolonged AIC with a constant need for blood products and refractoriness to other treatments (patients #2,5). One is alive and well, with normal counts (patient #2). The other, who rejected his 1st transplant and developed refractory AIC after his 2nd HSCT, died three weeks after his 3rd HSCT from sepsis and multi-organ failure (patient #5).

Novel treatment modalities for the more refractory cases are direly needed. Strategies to increase peripheral tolerance by enhancing the donor Treg engraftment may be the key (21), as illustrated in patients with IPEX (immune dysregulation, poly-endocrinopathy, enteropathy, x-linked) syndrome. The phenotype of these patients is caused by mutations in FoxP3, leading to a lack of functional Tregs. HSCT, specifically donor Treg engraftment, has been shown to cure these patients and demonstrates the impact of functioning Tregs on immune regulation (22, 23).

CONCLUSION

In conclusion, post-HSCT AIC presents with a variable presentation and a wide spectrum of severity. Some of these cases are prolonged and refractory, even requiring a repeated HSCT. In the rare group of MIOP patients, AIC is relatively prevalent, possibly due to the difficult engraftment and high incidence of mixed chimerism in these patients. There is a dire need for novel treatment modalities for better management of the more severe and refractory cases.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hadassah Medical Centers' Ethics Committee. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

EE-O and PS conceptualized and drafted the manuscript; YS, AN, IZ, and BS reviewed and edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version

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REVIEW
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Autoimmune Cytopenias in Common Variable Immunodeficiency Are a Diagnostic and Therapeutic Conundrum: An Update

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Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (PID). CVID is a heterogenous condition and clinical manifestations may vary from increased susceptibility to infections to autoimmune manifestations, granulomatous disease, polyclonal lymphoproliferation, and increased risk of malignancy. Autoimmune manifestations may, at times, be the first and only clinical presentation of CVID, resulting in diagnostic dilemma for the treating physician.

Autoimmune cytopenias (autoimmune haemolytic anaemia and/or thrombocytopenia) are the most common autoimmune complications seen in patients with CVID. Laboratory investigations such as antinuclear antibodies, direct Coomb's test and anti-platelet antibodies may not be useful in patients with CVID because of lack of specific antibody response. Moreover, presence of autoimmune cytopenias may pose a significant therapeutic challenge as use of immunosuppressive agents can be contentious in these circumstances. It has been suggested that serum immunoglobulins must be checked in all patients presenting with autoimmune cytopenia such as immune thrombocytopenia or autoimmune haemolytic anaemia.

It has been observed that patients with CVID and autoimmune cytopenias have a different clinical and immunological profile as compared to patients with CVID who do not have an autoimmune footprint. Monogenic defects have been identified in 10-50% of all patients with CVID depending upon the population studied. Monogenic defects are more likely to be identified in patients with CVID with autoimmune complications. Common genetic defects that may lead to CVID with an autoimmune phenotype include *nuclear factor kappa B subunit 1 (NF-kB1), Lipopolysaccharide (LPS)-responsive beige-like anchor protein (LRBA), cytotoxic T lymphocyte antigen 4 (CTLA4), Phosphoinositide 3-kinase (PI3K), inducible T-cell costimulatory (ICOS), IKAROS* and *interferon regulatory factor-2 binding protein 2 (IRF2BP2).*

In this review, we update on recent advances in pathophysiology and management of CVID with autoimmune cytopenias.

Keywords: common variable immunodeficiency (CVID), autoimmune cytopenia (AIC), B cells, lipopolysaccharide (LPS)-responsive beige-like anchor protein (LRBA), cytotoxic T lymphocyte antigen 4 (CLTA-4), B cell activating factor (BAFF), inducible T cell co-stimulator (ICOS)

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INTRODUCTION

Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency (1–3). CVID is a predominant antibody deficiency disease and there is marked reduction of serum immunoglobulin (IgG) and immunoglobulin (IgA) and/or immunoglobulin (IgM) along with impaired or poor response to vaccines (2). Since the first description of this entity in 1954 (4), there has been a remarkable progress in understanding the clinical phenotype of this disease. CVID is a heterogenous condition and clinical manifestations may vary from increased susceptibility to infections to autoimmune manifestations, granulomatous disease, polyclonal lymphoproliferation, and increased risk of malignancy. Autoimmune manifestations may be seen in 25 to 30% of all patients with CVID and may, at times, be the first and only clinical presentation (2). Such presentations of CVID can result in diagnostic dilemma for the treating physician.

Of the various autoimmune complications seen in patients with CVID, autoimmune cytopenias (autoimmune haemolytic anaemia, thrombocytopenia, Evan's syndrome, neutropenia and pernicious anaemia) are the most common (5). Laboratory investigations such as antinuclear antibodies, direct Coomb's test and anti-platelet antibodies may be negative in patients with CVID because of lack of specific antibody responses. Moreover, presence of autoimmune cytopenias may pose a significant therapeutic challenge as use of immunosuppressive agents can be contentious in these circumstances.

It has been observed that patients with CVID and autoimmune cytopenias have a different clinical and immunological profile as compared to patients with CVID who do not have an autoimmune footprint (5). Monogenic defects have also been identified in 10-50% of all patients with CVID depending upon the population studied (6). Monogenic defects are more likely to be identified in patients with CVID with autoimmune complications (7).

This review will elaborate on recent developments in pathophysiology and management of CVID in the context of autoimmune cytopenia.

CLINICAL PHENOTYPE OF AUTOIMMUNE CYTOPENIA IN CVID

Of the various autoimmune complications in CVID, cytopenia has been reported to be the most common complication (5). Recent data from United States Immunodeficiency Network (USIDNET) registry showed that patients with CVID with autoimmune cytopenia had one or more of disease associated non-infectious complications such as lymphoproliferation, liver disease, interstitial lung disease, granulomatous inflammation, enteropathy, other-organ specific autoimmunity and increased risk of lymphoma (8). This complex interplay between autoimmune manifestations in various systems remains an enigma and exact etiopathogenesis remains speculative. In one of the largest cohorts of CVID patients, Gathmann et al. reported a strong association between autoimmunity and enteropathy (9). Another study by Mormille et al. reported splenomegaly in almost all patients with cytopenia (88%) (10). Most studies over last two decades have shown an association of autoimmune cytopenia with splenomegaly and granulomatous disease (**Table 1**).

Immune Thrombocytopenic Purpura

Of the various autoimmune cytopenia in CVID, ITP is the most frequently reported manifestation by several authors (8–12, 14–17, 21, 24–26) Initial studies from United Kingdom (UK) and United States of America (USA) reported that autoimmune cytopenia is more common in females with CVID (11, 12). It was also opined that females tend to have a later onset of disease as compared to males. These studies, however, did not classify the effect of autoimmunity on different cell lineages and included cytopenia as a whole. In the USA cohort, it was observed that both serum IgA and IgM were higher in females and this finding was postulated to be a risk factor for autoimmunity (12). Another study by Kokron et al. found that although women had late-onset autoimmunity, the overall morbidity and mortality remained similar for both genders (14). This observation is similar to most other studies over last two decades that have shown that there is no gender predisposition to autoimmunity in patients with CVID (**Table 1**).

Proportion of patients with CVID who develop ITP has been reported to vary from 7.4 to 19% (**Table 1**). These differences could be attributed to the study design or hitherto unknown genetic differences in different ethnicities. Studies prior to 2000 have shown that most patients with CVID with ITP had mild symptoms (11, 12). Even in patients with clinically significant thrombocytopenia, splenectomy was not considered as a therapeutic option by most treating physicians. However, recently it has been noted that splenectomy may be considered in refractory cases of ITP. Splenectomy was not found to increase overall morbidity and mortality, provided that these patients were continued on regular intravenous immunoglobulin (IVIg) replacement (10, 12, 19, 25, 26).

At times, ITP may be the first and only symptom of CVID. A French study in 2004 included 21 patients with ITP who were also diagnosed to have CVID (15). Of these, most patients (62%) had delay in diagnosis of CVID (more than 6 months after the diagnosis of ITP). Only 19% patients were diagnosed to have CVID before the diagnosis of ITP. Another study from USA in 2005 has reported that most patients (54%) had ITP as the first manifestation of CVID (16). A large multicentric study from Europe also reported that presence of ITP often delays the diagnosis of CVID (21). Thus, it may be suggested to check serum immunoglobulins in all patients with ITP who often report to the haematology clinic.

Autoimmune Haemolytic Anaemia

Following ITP, the other most common autoimmune cytopenia in CVID has been reported to be AIHA (5). As in cases of ITP, women tend to have a later onset of disease, although the overall morbidity and mortality remained the same between the 2 genders (21). There is a wide variation in proportion of patients with CVID who have been reported to develop AIHA

TABLE 1 | Review of studies that have reported the clinical phenotype of autoimmune cytopenia in CVID.

Author, year, country	Number of patients [x/y][#]	Sex ratio (M: F)	Age at diagnosis (years)	Salient findings	Management
Hermaszewsky et al., 1993, UK (11)	40/240	NA	Biphasic (1-5; 16-20)	12 CVID patients had AIHA, 6 had ITP, 4 had pernicious anaemia and 18 had neutropeniaThrombocytopenia was mild and nearly half of these patients had splenomegalyNeutropenic patients had poor prognosis because of increased infections	Splenectomy was performed in 5 and 2 patients with AIHA and ITP respectively
Cunningham-Rundles et al., 1999, USA (12)	32/248	51:73 (15:17)	29 (Male)33 (Female)	Females had a higher predisposition for autoimmunity including cytopenia15 patients had ITP, 12 had AIHA, 3 had pernicious anaemia, 2 had autoimmune neutropenia, 5 had Evan's syndrome	IVIg and short course steroids
Kainulainen et a.l, 2001, Finland (13)	10/95	52:43	33	Eighteen (19%) patients with CVID had autoimmune manifestations; pernicious anaemia was the commonest (6%) followed by ITP (3%) and AIHA (1%)	NA
Kokron et al., 2004, Brazil (14)	3/71	38:33	15-78	2 patients had haemolytic anaemia, while 1 had pernicious anaemia; 1 female patient had both haemolytic anaemia and Sjoügren Syndrome and 1 male patient had atrophic gastritis and pernicious anaemia	IVIg
Michel et al., 2004, France (15)	21/21	4:3	27 (10-74)	The median age at AITP diagnosis was earlier than the diagnosis of CVIDCVID was diagnosed before the onset of AITP in only 4 patients (19%). It was diagnosed more than 6 months after AITP in 13 cases (62%), and the 2 conditions were diagnosed concomitantly in 4 cases11 patients (52%) had at least 1 autoimmune manifestation other than AITP, among which AIHA (7 cases) and autoimmune neutropenia (5 cases) were more common	The commonest treatment included steroids and IVIg (1-2g/kg). 6 patients needed additional therapy including azathioprine, vincristine and cyclophosphamide4 patients underwent splenectomy for AITP (2 had complete remission and 2 failed to respond). Two patients underwent splenectomy for Evans syndrome
Wang et al., 2005, USA (16)	35/326	16:19	5-66	19 (54%) patients had the 1st episode of thrombocytopenia or haemolytic anaemia prior to the diagnosis of CVID, 11 (32%) were diagnosed concurrently, and 5 (14%) developed one or both of these autoimmune diseases following the diagnosis of CVID; 8 patients with cytopenia also had granulomas	Treatment included corticosteroids, anti-Rh immunoglobulin, and intravenous immunoglobulinEleven patients underwent splenectomy
Carbone et al., 2006, Spain (17)	3/14	4:3	37.4(21-68)	2 patients had ITP and 1 had AIHA	NA
Alachkar et al., 2006, UK (18)	NA/34	25:9	25 (8-51)	Reduced switched memory B cells was associated with a significantly higher prevalence of bronchiectasis, splenomegaly and autoimmunity	NA
Quinti et al., 2007, Italy (19)	97/224*	48:49	26.6 (2-73)	At the time of diagnosis of CVID, autoimmune diseases were the only features in 2.3% of patients while in 11.1% autoimmune diseases were associated with recurrent infections	Steroids and splenectomy (more details NA)
Chapel et al., 2008, UK, Sweden, Germany, France, Czech Republic (20)	40/334	1.4:1	33	There was a statistically significant correlation of splenomegaly with cytopenias, hepatomegaly, and granulomata, but not with solid organ–specific autoimmunity	NA
Wehr et al., 2008, UK, Germany, France, Spain, Netherlands and Czech Republic (21)	43/303	133:169	35 (3-74)	The age of onset of immunodeficiency was delayed in CVID patients with autoimmune manifestations although it was not statistically significant because of low numbers; majority had ITP (64%), followed by AIHA (25%), and 11% had Evan's syndrome; nine patients had pernicious anaemia; There was no difference between genders; autoimmune cytopenia had significant associations with splenomegaly and granulomatous disease	NA
Ardeniz et al., 2009, Turkey/ USA (22)	19/37	13:24	26 (2-59)	7 patients with autoimmune cytopenia also had granulomas (lung and liver) as the predominant manifestation	Steroids used most commonly; 2 patients received cyclosporin, 1 infliximab and 1 rituximab
Mouillot et al., 2010, France (23)	55/313	0.9:1	45 (33-56)	Correlation was noted between decreased switched memory B cells, decrease in naive CD4+ T cells and increase in CD4+CD95+ cells with lymphoproliferation, autoimmune cytopenia, or chronic enteropathyIn addition, lymphoproliferation and cytopenia patients had increase in CD21low B cells and CD4+HLA-DR+ T cells and decreased regulatory T cell	NA
Boileau et., 2011, France (24)	55/311	29:26	29 (16-46)	41 patients (74%) had ITP, 17 patients (31%) had AIHA and 10 patients (18%) had neutropenia. 36 patients in this group developed splenomegaly (65%) and 8 patients developed a granulomatous	NA

(Continued)

TABLE 1 | Continued

Author, year, country	Number of patients [x/y][#]	Sex ratio (M: F)	Age at diagnosis (years)	Salient findings	Management
				disease (14%); a significant correlation was found between an increased proportion of CD21low B cells and CVID associated autoimmune cytopenia; in CVID associated autoimmune cytopenia, T cells display an activated phenotype with an increase of HLA-DR and CD95 expression and a decrease in the naïve T cell numbers	
Maarschalk-Ellerbroek et al., 2012, Netherlands (25)	9/61	25:36	27 (14-43)	At diagnosis, 3 patients had cytopenia (AIHA/ITP), and it increased to 9 at follow-up (median 7 years); splenomegaly seen in 8 patients; low switched memory B cells associated with autoimmunity, splenomegaly and granulomas	NA
Arshi et al., 2016, Iran (26)	21/47	1:1	27 (4-63)	ITP was the commonest manifestation (26%) followed by AIHA (15%) and pernicious anaemia (4%)Autoimmunity occurred in older age group (mean 14.2 years) and was associated with parental consanguinity (57%)	IVIg in all and splenectomy in 3 patients with ITP
Patuzzo et al., 2016, Italy (27)	10/10	1:4	44.8 (±12)	Patients with CVID and AITP had a higher percentage of CD21low cells	NA
Arduini et al., 2016, Ireland (28)	2/23	13:10	22-82	1 patient had AIHA, ITP and neutropenia; 1 patient had pernicious anaemiaPeripheral mucosal-associated invariant T cell activation is a feature of CVID and depletion of these cells is particularly associated with complications including autoimmunity	NA
Çalişkaner et al., 2016, Turkey (29)	3/25	12:13	36.6 (± 13.4)	3 patients had ITP (2 had splenomegaly and 1 required splenectomy)	IVIg and steroid
Almejun et al., 2017, Argentina (30)	5/25	12:13	11.3 (4-16.1)	Severe altered somatic hypermutation in addition to low switched memory B cells has a correlation with autoimmunity, splenomegaly and granulomas	NA
Feuille et al., 2017, USA (USIDNET Registry) (8)	101/990	52:49	16 (10-31)	The most common autoimmune cytopenia was ITP (N = 73), followed by haemolytic anaemia (N = 45), and autoimmune neutropenia (N = 10); There was no significant difference in the age at diagnosis, gender, and baseline Ig values between the group with autoimmune cytopenia and those without cytopenia; autoimmune cytopenia group was more likely to have lymphoproliferation, granulomatous disease, lymphomas, hepatic disease, interstitial lung diseases, enteropathy, and organ-specific autoimmunity	NA
Guffroy et al., 2017, France (31)	16/473	1.7:1	17 (4-63)	Frequency of neutropenia 3.4%.16 patients had neutropenia and 11 of them were AINFive patients died during the follow-up (11 years) with an increased percentage of deaths in patients with neutropenia	Specific treatment for neutropenia was in general not administered, except in 3 patients who received G-CSF
Alkan et al., 2018, Turkey (32)	2/12	7:5	11.6 (± 3.7)	2 patients had Evans syndrome and splenomegalyBoth patients with cytopenias were diagnosed after 10 years	NA
Ghorbani et al., 2019, Iran (33)	18/220	1.2:1	9.5 (3.9-18.25) 5 (1.8-10)**	Frequency of neutropenia was 8.1%; Candida infection and septicaemia were significantly higher in neutropenic patients; the most prominent clinical phenotypes of CVID patients with neutropenia were polyclonal lymphocytic infiltration and autoimmunityThe mortality rate in neutropenic patients was higher than in patients without neutropenia (61.1 vs. 25.2%, p=0.004)	IVIg and prophylactic antibiotics for neutropeniaG-CSF and splenectomy were considered in 1 and 2 patients respectively
Mormille et al., 2021, Italy (10)	17/95	9:8	24-76	The most common autoimmune manifestation was cytopenia (17.8%); the most common cytopenia was immune thrombocytopenia, reported in 10 out of 95 patients (10.5%), followed by autoimmune haemolytic anaemia (n=3, 3.1%) and autoimmune neutropenia (n=3, 3.1%); almost all patients with autoimmune cytopenia had splenomegaly (15 out of 17; 88%)There was no statistically siginificant difference in CD3+, CD8+, CD4+CD25highCD127low T reg, CD19, CD19hiCD21loCD38lo, and follicular T helper cells in CVID patients with or without autoimmune manifestations	IVIg and steroid1 patient underwent splenectomy

NA, not available; CVID, common variable immunodeficiency; IVIg, Intravenous immunoglobulin; G-CSF, Granulocyte colony stimulating factor.

[#]x: no. of autoimmune cytopenia patients, y: total no. of CVID patients.

**97 patients had autoimmune manifestations (exact number of patients with autoimmune cytopenic not reported).*

***Neutropenic patients with CVID.*

(between 1 to 15%) (**Table 1**). In one study involving 326 patients with CVID, cytopenia was seen in 11% (n = 35): 9 had AIHA, and 11 had Evans syndrome (16). Most patients developed autoimmune cytopenia before or concurrent with the diagnosis of CVID. A similar observation has also been reported by several other authors. It may also be suggested to test for serum immunoglobulins in all patients who have AIHA.

Polyautoimmunity has been reported in as high as one-third of all patients with CVID who have autoimmune cytopenia (34). Although various other organ systems may be involved, the commonest association of AIHA is with ITP (Evans syndrome) (34). A multicentric study by Wehr et al. observed that Evans syndrome was seen in 11% patients with CVID who had autoimmune cytopenia (21). Besides, both AIHA and ITP may occur concomitantly with autoimmunity in other organ systems including gastrointestinal, endocrine, rheumatological and dermatological. A recent meta-analysis has shown that haematological autoimmunity coexists with gastrointestinal and rheumatological autoimmunity in 3.1% and 2.1% patients respectively (34).

Autoimmune Neutropenia

There may be several causes of neutropenia in CVID. These include infection/sepsis induced, drug related, sequestration by spleen, autoimmunity or paradoxical neutropenia following IVIg infusion (31, 33). Most published literature on neutropenia in CVID is in the form of case reports and case series. These studies have reported neutropenia in <1% to 4% of all CVID patients (**Table 1**). An Iranian study observed neutropenia in 8.1% of all patients with CVID (33). However, in this cohort, all causes of neutropenia were included.

Similar to ITP and AIHA, there is no significant gender difference in the proportion of patients who develop AIN. However, in contrast to other forms of autoimmune cytopenia, patients with AIN are diagnosed early and the diagnosis of AIN rarely antedates the diagnosis of CVID (31, 33).

Polyautoimmunity is also commonly seen with AN and the most frequent associations are with ITP and AIHA (19). Ghorbani et al. also reported rheumatoid arthritis, vitiligo and autoimmune hepatitis in association with AIN (33).

There has been a frequent association of infections with AIN. However, whether this infection causes neutropenia or neutropenia per se is because of autoimmunity and is contributing to infections, remains contentious. In a study from Iran, fungal infections such as candidiasis and pancytopenia (27.5%) were observed more commonly in patients with neutropenia (33). Another study from French DEFI cohort reported that patients with AIN have unusual opportunistic infections such as *Pneumocystis sp*, deep mycotic infections, cryptosporidium, aspergillosis and cytomegalovirus colitis (31).

Patients with CVID and AIN have been reported to have poor prognosis. Ghorbani et al. reported a higher frequency of deaths (61.5%) in their cohort of patients with CVID who had AIN (33). Another study reported an eight-year overall survival rate of 50% in patients with AIN as compared to 87.5% survival rate in non-neutropenic patients (31). Thus, neutropenia in patients with CVID warrant prompt investigation and initiation of appropriate therapy as it may have an impact on overall mortality.

Pernicious Anaemia

Pernicious anaemia has been reported to be the least common amongst the various autoimmune cytopenia associated with CVID (**Table 1**). There are no large studies on PN in CVID. In a recent meta-analysis, the prevalence of PN was reported to be 2.4% (95% CI) (34).

Although PN has been described in literature since the 1840s, however, PN in the context of CVID was first described in 1969 (35). Most authors have defined classical PN as the presence of: "(1) Haemoglobin concentration < 13 g/dL for men and <12 g/dL for women, (2) red blood cell's mean corpuscular volume ≥ 120 fL, (3) low levels of serum vitamin B_{12}, (4) gastric body mucosal atrophy, and (5) auto-antibodies to intrinsic factor and/or to gastric parietal cells" (36). However, PN in CVID has been described in association with achlorhydria, atrophic gastritis, absence of intrinsic factor, absence of antibodies to gastric parietal cells and intrinsic factor, and malabsorption of vitamin B_{12} (37, 38). This entity may, at times, be difficult to differentiate from classical pernicious anaemia. However, PN in CVID usually occurs early and has low to absent autoantibodies, and presence of atrophic gastritis without plasma cell infiltrate in the lamina propria (38). The pathogenesis remains unexplained although it has been hypothesized that this subset of patients with CVID may have additional T-cell defects (37).

PATHOGENESIS OF AUTOIMMUNE CYTOPENIA IN CVID

Mechanism of autoimmunity in CVID remains an enigma. Both innate and adaptive arms of the immune system have been found to play a role in the pathogenesis of autoimmunity in CVID including autoimmune cytopenia (3). **Table 2** lists the salient findings of various studies that have reported immune abnormalities associated with autoimmune cytopenia in patients with CVID.

ROLE OF DYSREGULATED B CELLS IN CVID ASSOCIATED AUTOIMMUNE CYTOPENIA

Autoimmunity in patients with CVID is a complex pathophysiological mechanism as it represents a state of overreactive immune system in an otherwise immunocompromised host. Impairment in the development and function of B cells is a hallmark of CVID. Most patients with CVID have normal peripheral B cell counts and reduced $CD27^+$ memory B cells with severely impaired capacity to produce antibodies. A proportion of patients with CVID, however, tend to produce autoantibodies against self-antigens (44).

Studies have shown that development of autoimmune cytopenia in CVID is linked to a lower efficacy of the self-

TABLE 2 | Review of studies that have reported the immunopathogenesis of autoimmune cytopenia in CVID.

Author, year, country	Title	N	Technique used	Salient Features
G. Azizi et al., 2017; Iran (3)	Autoimmunity and its association with regulatory T cells and B cell subsets in patients with common variable immunodeficiency	72	Flowcytometric evaluation of T and B cell compartment	Higher transitional MZ B cells in patients with CVID with autoimmune cytopeniaLower percentage of naive and non-class-switched memory B cells were seen in patients with CVID with autoimmunityPatients with CVID with multiple autoimmune syndromes had higher level of CD3+ T cells, CD4+ T cells and CD21low B cells and lower number of T regs and naïve B cell when compared with patients with CVID with one autoimmune syndrome
Warnatz et al., 2002; Germany (39)	Severe deficiency of switched memory B cells (CD271IgM2IgD2) in subgroupsof patients with common variable immunodeficiency: a new approach toclassify a heterogeneous disease	38 (30 CVID; 22 HC)	Flowcytometry	Reduced class switched memory B cells (<0.4%) and increased CD21low B cells (>20%) in patients with autoimmune cytopenia
E. Kofod-Olsen et al; 2016., Denmark (40)	Altered fraction of regulatory B and T cells is correlated with autoimmune phenomena and splenomegaly in patients with CVID	34; 11 (HC)	Flowcytometry: Intracellular IL-10 expression analysisIntracellular FoxP3 expression analysisT cell suppression assay	Pronounced Reduction in Tregs in patients with CVID with autoimmunityrTregs (resting) were significantly reduced in the autoimmunity grouppatients had a significant reduction in CTLA-4 expression in all subsets except the rTregsSignificantly high expression of pro-B10 cells in autoimmunity group
Genre et al., 2009; Brazil (41)	Reduced frequency of CD4 +CD25HIGHFOXP3+ cells and diminished FOXP3 expression in patients with Common Variable Immunodeficiency:A link to autoimmunity?	33, 30 (HC)	Flow cytometric analysisRT PCR of FOXP3	Decrease of absolute CD4+ lymphocytes numberslower frequency of CD4 +CD25HIGHFOXP3+ cells in patients with AI with CVID than without AIReduced FOXP3 mRNA levels in Tregs of patients with CVID (Higher Reduction in AI+CVID group)
Tahiat A et al., 2014; Algeria (42)	Common variable immunodeficiency (CVID): clinical and immunological features of 29 Algerian patients	29	Flowcytometry	Decreased circulating B (54.2%) and T CD4+ (41.7%) cells and inversion of the CD4/CD8 ratio (70.8%). Patients with decreased circulating B and T CD4+ cells were significantly more likely to have auto-immune cytopenias and lymphoproliferative disease.
Mouillot G, et al., 2010 (23);	B-Cell and T-Cell Phenotypes in CVID Patients Correlate with the Clinical Phenotype of the Disease.	313; 50 (HC)	Flowcytometry	Reduced smB cells, Increased $CD21^{low}$ B cellsSignificant reduction in activated T cells ($CD4^+$and $CD95^+$ T cells) (AC>IO group)Reduced CD4+ and $HLADR^+$ T cells and Tregs
Romberg et al., 2019 (43)	CVID patients with autoimmune cytopenias exhibit hyperplastic yet inefficient germinal centre responses	14 CVID +AIC and 4 CVID-AIC patients.	Flowcytometry *In Vitro* T suppression activity, Lymph node staining and RT PCR	CVID+AIC patients displayed irregularly-shaped, hyperplastic germinal centres (GCs), whereas GCs were scarce and small in CVID-AIC patients evidenced by an increase in circulating T follicular helper cells, which correlated with decreased regulatory T cell frequencies and function

MZ, Marginal Zone; RT PCR, Polymerase chain reaction; Tregs, regulatory T cells; HC, Healthy controls; rTregs, Resting regulatory T cell; aTregs, Activated regulatory T cells; CTLA4, Cytotoxic T lymphocyte associated protein 4; pro-B10, regulatory B cells; AI, Autoimmunity; smB, Switched memory B cells; AIC, Autoimmune cytopenia; GC, Germinal Centre; IO, Infection Only.

tolerance mechanisms thereby leading to an altered immune-regulation (8). It has been observed that patients with CVID with autoimmune cytopenia may have characteristic abnormalities in the B cell immunophenotyping. These patients have been reported to have significantly reduced numbers of CD19+ B cells as compared to patients with CVID who do not develop autoimmune cytopenia (45). The most striking abnormality, however, is the expansion of an unusual population of B cells that lack complement receptor 2 (CR2/CD21) [$CD21^{lo}$ B cells] (45). $CD21^{lo}$ B cells represent a pool of autoreactive B cells. Autoreactive B cells are generated during random process of V (D)J recombination. Autoreactive B cells are generally silenced by 3 main mechanisms: deletion, receptor editing, and anergy. Receptor editing and deletion results in central tolerance that omits autoreactive immature B cells. However, a small percentage of autoreactive B cells escape the bone marrow and remain in periphery where anergy renders them unresponsive to antigenic stimuli (46). Important immune abnormalities have been illustrated in **Figure 1**.

$CD21^{lo}$ B cells manage to escape the central B-cell tolerance and remain in the periphery in an unresponsive stage. Low proportion of these cells are also present in healthy individuals. $CD21^{lo}$ B cells have short life span and are usually eliminated in normal individuals. However, various studies have shown an expansion of $CD21^{lo}$ clones of autoreactive B cells in patients with systemic lupus erythematosus (SLE), CVID and rheumatoid arthritis (RA) (47). Factors that favour maintenance and survival of the autoreactive and unresponsive $CD21^{lo}$ B cells in the

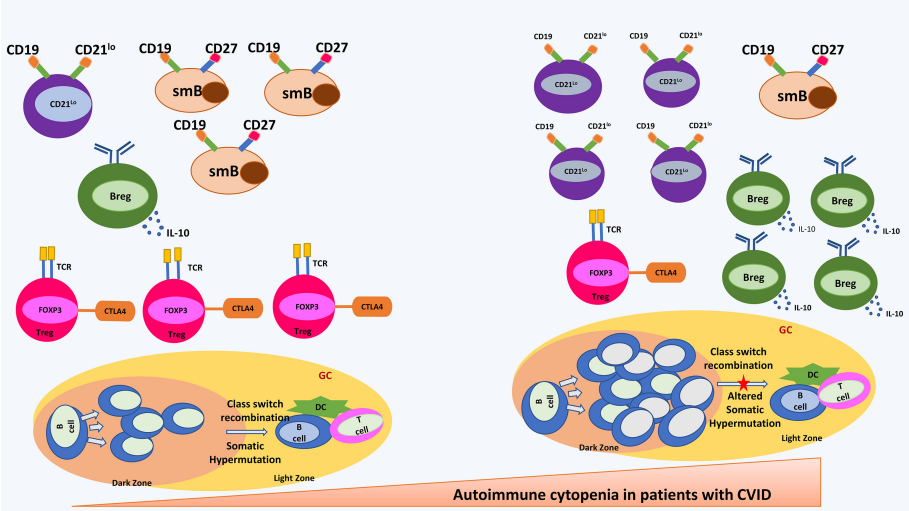

FIGURE 1 | shows most important immune abnormalities that have been reported in patients with CVID with autoimmune cytopenia. Shown in the right panel are an increased CD21^{lo} B cell and B regulatory cells; decreased switched memory B cells and T regulatory cells; hyperplastic germinal centre and altered somatic hypermutation. CD21^{lo}: CD21$^{-/LOW}$ B cells; smB, switched memory B cells; Breg, B regulatory cells; Treg, T regulatory cells; DC, Dendritic Cells; GC, germinal centre.

peripheral circulation of patients with RA and CVID are unknown. It has been suggested that elevated concentrations of B cell activating factor (BAFF) in the serum of patients with CVID lead to inhibition of removal of anergic CD21^{lo} B cells from periphery (48). Presence of anergic CD21^{lo} B cells with low avidity to bind to self-antigens pose a major risk of development of autoimmunity. Murine studies have shown that inactive CD21^{lo} B may overcome the state of anergy during infection where cross-reactive antigenic epitopes present on infectious agents stimulate anergic B cells *via* innate immune ligands (49).

Isnardi et al. studied the pool of CD21^{lo} B cells in patients with CVID and RA. CD21^{lo} B cells were found to be elevated in these patients. It was further observed that these CD21$^{-/lo}$ B cells are closer to the naïve B cell population (in comparison to the isotype switched CD21^{lo} B cells seen in healthy individuals) and express germline B-cell receptor (BCR) repertoire that is rich in autoreactive clones. Immunofluorescence assay showed that CD21^{lo} B cells expressed antinuclear antibodies (speckled nuclear and nucleolar pattern) and also expressed autoantibodies against several cytoplasmic structures. CD21^{lo} B cells do not get activated and do no proliferate through BCR and CD40 co-stimulation and showed impaired calcium-mediated signalling. This results in inactivation of a few activation markers on B cells upon BCR triggering. Impaired activation has been linked to impaired proliferation of B cells to antigenic stimuli suggesting their unresponsive stage. The transcriptome analysis of CD21^{lo} B cells revealed up-regulation of several genes implicated in the inhibition of B-cell activation, proliferation, and survival and the downregulation of B-cell activating genes, suggesting an inhibitory gene signature. These results were further confirmed using flow cytometry assays that suggested downregulation of receptors that favour B cell survival and upregulation of receptors that favour B cell inhibition. In addition, the survival potential of CD21^{lo} B cells was compared with that of CD21^{+} B cells and it was found that CD21^{lo} B cells were prone to die by apoptosis suggesting they have a shorter half-life (47).

Warnatz et al. classified CVID patients with low CD27^{+} B cells into 2 groups based on proportion of CD21$^{\mathbf{lo}}$ B cells. Group Ia had more than 20% CD21$^{\mathbf{lo}}$ B cells and these patients were found to be more susceptible to develop autoimmune cytopenia (and not the other autoimmune manifestations) (39).

The French DEFI group screened 311 patients with CVID and divided them (based on the clinical manifestations) into non infection (NI), autoimmunity (AI) and autoimmune cytopenia (cy) group. Absolute numbers of B and T cells were low but comparable among the 3 groups. However, a significant association between CD21^{lo} B cells and autoimmune cytopenia was reported. Percentage increase in CD21^{lo} B cells was comparable in NI and AI group while it was significantly high in the cy group. This suggests that higher proportion of CD21^{lo} B cells in patients with CVID correlate specifically with an increased risk of autoimmune cytopenia (23).

Role of pro B10 cells (also known as regulatory B cells [Bregs], identified by the production of IL-10 cytokine) has been reported in a number of immune mediated disorders, such as RA and ITP. Olsen et al. reported that pro B10 cells were increased in patients with CVID who developed autoimmunity and splenomegaly whereas patients with CVID without autoimmunity displayed only a modest increase in these cells (40). The underlying mechanistic link between elevated pro-B10 cell levels and autoimmunity in CVID patients is not clear at present. However, contrasting results have been reported in murine models and patients with RA. Proportions of Bregs have been

reported to be reduced and inversely related to disease severity in patients with RA. This fraction of B cells has been reported to be increased in patients with ITP and patients with chronic hepatitis. It has been hypothesized that increase in Bregs in patients with CVID suggest a compensatory mechanism wherein the Bregs expand to compensate for reduced level of regulatory T cells (Tregs) and this effect seems to be more pronounced in patients with autoimmunity and splenomegaly (43).

Romberg et al. compared the germinal centre (GC) responses of patients with CVID with autoimmune cytopenia (CVID+AIC) and CVID patients with autoimmunity other than cytopenia (CVID-AIC). Irregularly shaped and hyperplastic germinal centres along with increased number of circulating T follicular helper cells were observed in CVID+AIC group while GC structure in CVID-AIC group were found to be small and circular. CVID+AIC cohort had higher CD19hiCD21-/lo B cells compared to CVID-AIC cohort, CD27+IgG+ memory B-cell population and IgA+ B cells were reduced in CVID+AIC group (43).

The study also evaluated somatic hypermutation (SHM) in CD27+IgG+ memory B-cells. Patients with CVID were found to have lower SHM frequencies in heavy chain variable regions (VH) than controls. CVID+AIC patients showed least SHM (7.5 mutations per VH segment) as compared to 15.1 in CVID-AIC group and 18.6 in heathy controls. VH4-34 gene segment was identified in 9.9% of CVID+AIC IgG transcripts while this segment was rarely seen in CVID-AIC group and healthy controls. VH4-34-encoded antibodies have been found to be autoreactive as they bind the conserved I/i carbohydrate self-antigens expressed in red blood cells and other hematopoietic cell lineages (43).

Yu et al. have also reported that patients with autoimmunity with CVID had significantly reduced switched memory B cells (50).

ROLE OF DYSREGULATED T CELLS IN CVID ASSOCIATED AUTOIMMUNE CYTOPENIA

Role of T cell compartment in the development of autoimmune cytopenia in patients with CVID has also been reported by several authors. Disturbed T cell homeostasis underlies the pathogenesis in one third of CVID patients with autoimmune manifestations. These include alterations in number of CD4, CD8 T cells, memory T cells, regulatory T cells, and altered expression of transcripts essential for regulatory T cells functioning.

The French DEFI group study reported reduced number of switched memory B cells and naïve $CD4^+$ T cells in CVID associated autoimmune cytopenia. This reduction was accompanied by activated phenotype with an increased expression of HLA-DR and CD95 markers on $CD4^+$ T cells. Patients with other autoimmune manifestations did not show this T and B cell phenotype (23).

In another study on 29 patients with CVID, abnormality in T and B cell phenotypes was detected in 75% cases, mostly reduced circulating B cells (54.2%) and CD4+ T (41.7%) cells. There was inversion of CD4/CD8 ratio (70.8%). Patients with decreased circulating B and CD4+ T cells were significantly more likely to have auto-immune cytopenias and lymphoproliferative disease (42).

It has also been reported that patients with CVID with autoimmune manifestations have significantly reduced proportion of CD8+ T cells (51).

Herrera et al. compared the absolute numbers of T, B and NK cells among patients with CVID. Lymphocyte profiles were compared between patients with CVID with autoimmunity and patients with CVID without autoimmunity and healthy controls. CD4+ T cell numbers in patients with CVID without AI were significantly lower compared with the control group. Patients with CVID with AI had increased CD4+CD45RO+ memory T cell populations compared with healthy controls (45).

Bateman et al. reported that naïve $CD4^+$ and $CD8^+$ T cell numbers were significantly reduced in patients with CVID especially in association with autoimmune cytopenia. Further, within CD4+ T cell compartment, there was reduction in $CD4^+CD45RA^-CCR7^+$ central memory T cells in autoimmune cytopenia group. In CD8+ T cells, $CD8^+CD45RA^-CCR7^-$ effector memory T were reduced in patients with CVID with organ specific autoimmunity and increased in patients with CVID with autoimmune cytopenia.

Enumeration of early differentiation stages of CD4+ and CD8+ T cells defined by co-expression of CD27/28 molecules revealed reduced numbers in autoimmune cytopenia and organ specific autoimmunity subgroups of patients with CVID. Reduction in the population of CD4+ and CD8+ T cell numbers was not accompanied by an increase in the numbers of recent thymic emigrants, suggesting a lack of replenishment of the lymphocyte pool from thymus (51).

In addition to these abnormalities various studies have highlighted the role of Tregs in patients with CVID with autoimmune manifestations. Tregs play pivotal role in limiting the persistent immune activation. Reduced counts along with impaired suppressive capacity of Tregs has been reported in literature

Freiburg classification differentiates patients with CVID into groups Ia and Ib with significantly lower percentages of Tregs compared to patients in group II and healthy controls. Autoimmune disease was found to be significantly higher in group Ia (52).

Horn et al. reported reduced number of Tregs in patients with CVID with immune cytopenia and granulomatous diseases (53).

A negative correlation between reduced Treg numbers and presence of autoimmunity in patients with CVID with AI has also been reported (45) (51).

Olsen et al. reported altered proportions of regulatory lymphocytes in patients with CVID. Patients with autoimmunity had reduced levels of resting Tregs and activated Tregs predominantly seen in patients with autoimmunity and splenomegaly. The impaired functioning of activated Tregs was further indicated by reduced expression of cytotoxic T lymphocyte antigen 4 (CTLA-4) on its surface (40).

Reduced number of Tregs and an increase in T follicular helper CD4+ cells has been reported in patients with CVID with

autoimmune cytopenia (41). Genre et al. reported compromised homeostasis of Tregs in a subset of patients with CVID with autoimmunity. Flowcytometry revealed reduced proportion of $CD4^{+}CD25^{HIGH}FOXP3^{+}$ Tregs in patients with CVID with autoimmunity as compared to patients with CVID without autoimmunity. Forkhead box P3 protein (FOXP3) mRNA (messenger ribonucleic acid) expression was also found to be reduced in patients with CVID compared to healthy controls and the reduction was more pronounced in patients who had autoimmune cytopenia (41).

Yu et al. reported Tregs dysfunction in patients with CVID with autoimmunity. Switched memory B cells and Tregs were found to be low along with reduced ability to suppress proliferation of autologous and allogenic CD4+ effector cells in patients with CVID with autoimmunity when compared with patients with CVID without autoimmune disease, healthy controls and disease control (patients with X-linked agammaglobulinemia). The key proteins involved in functioning of Treg, including FoxP3, Granzyme A, XCL1 (lymphotactin), pSTAT5 (phosphorylated signal transducer and activation of transcription-5 protein), and GITR (glucocorticoid induced tumor necrosis factor receptor related protein) were found to be significantly reduced in patients with CVID with autoimmunity. Results suggest that these proteins may be involved in Treg-mediated autoimmunity in patients with CVID (50).

DYSREGULATION OF INNATE IMMUNE SYSTEM IN CVID ASSOCIATED AUTOIMMUNE CYTOPENIA

Although defects in innate immunity have been reported in patients CVID, their correlation with autoimmunity has not been investigated in detail. Taraldsrud et al. and Sharifi et al. studied the role of Toll-like receptors (TLR) in the pathogenesis of CVID (54, 55). It was suggested that defective TLR7, TLR8, and TLR9 signalling may lead to dysregulation of self-tolerance and expansion of auto-reactive B cells.

Rezaei et al. measured various cytokines, especially type I interferons (IFN), in patients with CVID. It has been postulated that increased IFN-α/β may result in dysregulation of peripheral tolerance by activating immature dendritic cells (56). They may also lead to activation of autoreactive T cells, that in turn would increase autoreactive B cells and subsequent autoimmunity. However, evidence is still lacking and further studies are needed on this aspect (57).

PATHOPHYSIOLOGY OF NON-CVID ASSOCIATED AUTOIMMUNE CYTOPENIA

Autoimmune cytopenia may also be seen in several other disorders such as inborn errors of immunity (e.g. Wiskott-Aldrich syndrome, autoimmune lymphoproliferative syndrome, X-linked lymphoproliferative syndrome, severe combined immunodeficiency and complement defects), acquired causes such as (lymphoproliferative disorders, malignancies, systemic lupus erythematosus [SLE], drugs, infections and complication of organ or hematopoietic stem cell transplant). The pathophysiology of autoimmune cytopenia in several of these disorders especially those associated with inborn errors of immunity is more complex and similar to the mechanisms associated with CVID. On the other hand, the pathophysiology of autoimmune cytopenia in acquired disorders such as SLE or drug induced cytopenia is primarily associated with generation of auto-antibodies [e.g. autoantibodies against receptors on the platelet surface, GPIb/IX complex (vonWillebrand factor receptor) and the GPIIb/IIIa receptor (collagen/fibrinogen receptor) may be associated with autoimmune thrombocytopenia in SLE and antibodies against red blood cells may be associated with warm-reactive (W-AIHA), cold-agglutinin (C-AIHA), or paroxysmal cold haemoglobinuria (PCH)] (58, 59).

Autoimmune cytopenia in patients with autoimmune lymphoproliferative syndrome is primarily due to defective apoptosis of lymphocytes mediated through the Fas/Fas ligand pathway (60). On the other hand, pathophysiology of autoimmune cytopenia in context of complement defects is associated with defective clearance of apoptotic bodies and formation of immune complexes (59).

Genetic Link to Autoimmune Cytopenia in CVID

Monogenic defects have been identified in a small proportion of patients with CVID. These monogenic defects may have an important pathophysiological link with autoimmune cytopenia.

TNFESF13B gene encodes for TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor), a member of tumour necrosis factor receptor superfamily expressed on B cells. TACI has been found to play important role in the B cell development. Monoallelic heterozygous and biallelic (compound heterozygous and homozygous) defects in the gene encoding for TACI have been reported to cause CVID. However, there are speculations that TACI defects are diseases modifying rather than disease causing because several healthy individuals have been reported to have same defect but do not develop any clinical manifestations. A recent study from Greece reported that monoallelic defects in TACI may act as susceptibility or disease modifying factor in the pathogenesis of CVID. It was, however, observed that patients with CVID with TACI defects had significantly higher risk of autoimmune cytopenia as compared to patients with CVID without any TACI defect (61). In addition, studies have shown that TACI plays an important role in central B cell tolerance and defects in TACI lead to impaired central B cell tolerance leading to an increased production of autoreactive B cells (62). It is intriguing to note that patients with CVID with TACI defects and not the carriers of TACI defects are more prone to develop autoimmunity. The likely explanation for this is that patients with CVID have defect in peripheral B cells tolerance while this is not seen in healthy individuals with TACI defects. As a result, patients with CVID

with TACI defects are unable to compensate for loss of central B cell tolerance which is well compensated in healthy carriers of TACI defect. Moreover, the heterozygous monoallelic variants rather than biallelic variants are more likely to produce autoimmunity. This is because a more profound defect in TLR pathway defects in patients with CVID with biallelic variants in TACI provides protection against development of autoimmunity even though autoreactive B cells are also increased in patients with biallelic variants in TACI (63).

Tumor necrosis factor receptor superfamily member 13C (TNFRSF13C) encodes for BAFF-R (B-cell activating factor receptor) that functions as a pro-survival factor for B cells. Variants in BAFF-R lead to arrest of developing B cells at immature/transitional B cells stage. Similar to the TACI defect, the variants in BAFF-R may possibly be disease modifying rather than disease causing. A few patients with BAFF-R deficiency have been reported to develop autoimmune manifestations (6). The exact pathogenesis is not known but could be related to elevated serum BAFF levels because of BAFF-R deficiency (64). BAFF, which belongs to the TNF-ligand family, plays crucial role in B cell development, maintenance of auto-reactivity, and homeostasis (65, 66). Plasma BAFF levels have been found to be elevated in autoimmune disorders including SLE (67), RA (68), Sjögren syndrome (SS) (69). BAFF has also been found to be elevated in active ITP and levels normalise during remission (70). Elevated levels of BAFF promote the survival of auto-reactive B cells (71) and may lead to autoimmune cytopenia in CVID.

Inducible T cell costimulator (ICOS), a member of CD28/CTLA-4 family, plays important role in regulating T cell responses. ICOS deficiency was the first identified genetic defect in patients with CVID. ICOS ligand is expressed in monocytes, dendritic cells and B cells. In addition to hypogammaglobulinemia and recurrent infections, these patients have also been reported to develop autoimmune manifestations especially autoimmune neutropenia (72, 73) (74). The exact pathophysiology of autoimmunity in ICOS deficiency is not known. However, it has been suggested that decreased production of IL-10 and decreased expression of CTLA-4 in patients with ICOS deficiency is responsible for autoimmune manifestations (72). In the original description of ICOS deficiency in context of CVID, patients with autoimmune neutropenia were detected to have IgG antineutrophil antibodies, suggesting an ICOS independent class switch in these patients (73).

Lipopolysaccharide-responsive and beige-like anchor (LRBA) protein encoded by the *LRBA* gene, is a critical protein involved in the expression and intracellular trafficking of CTLA4 protein. Costimulatory signal between T cells and antigen presenting cells (APCs) using CD28 (on T cells) and CD80/86 on APCs is crucial in the activation of T cells. CTLA-4 has higher affinity for CD80/86 and outcompetes CD28 in binding to CD80/86, CTLA-4, therefore, constitute an important immune check-point by preventing overactivation of T cells. CTLA-4 is an important mechanism by which Tregs exert their inhibitory effect on activated T cells. Patients with homozygous or compound heterozygous variants in *LRBA* gene and heterozygous variants in *CTLA-4* gene fail to express CTLA-4 protein on surface and have been reported to develop CVID phenotype with autoimmunity, lymphoproliferation and inflammation (75). LRBA deficiency is one of the commonest genetic defects identified in patients with CVID (76).

Patients with LRBA deficiency and CTLA-4 haploinsufficiency present with a broad and overlapping clinical phenotype. Most common autoimmune manifestation in both these disorders include autoimmune cytopenia (seen in more than 2/3rd of all cases) (71, 73). LRBA deficiency and CTLA-4 haploinsufficiency leads to a normal or elevated number of Treg cells in the circulation. However, the Treg cell functions are impaired.

It has also been reported that monogenic defects may be identified in more that 2/3rd of all patients with Evans syndrome (autoimmune haemolytic anaemia and thrombocytopenia) especially defects in *LRBA* and *CTLA-4* gene. Other genetic defects reported in patients with Evans syndrome include heterozygous loss of function mutation in *TNFRSF6* gene, *CBL* gene and *ADAR1* gene; heterozygous gain of function mutations in *STAT3* gene and *PIK3CD* gene; and compound heterozygous mutations in *RAG1* gene. In addition, somatic mutations in *TNFRSF6* and *KRAS* genes and possibly pathogenic variants in several other genes were also reported. Patients with Evans syndrome who had a monogenic defect were more likely to have hypogammaglobulinemia and lymphoproliferation as compared to the patients with Evans syndrome who had no monogenic defects (77, 78).

BCR complex is composed of CD19, CD21, CD81 and CD225. Monogenic defects in CD19, CD21 and CD81 have been reported to lead to CVID phenotype. Of these, patients with CD19 and CD81 deficiency have also been reported to develop autoimmunity and autoimmune cytopenia have been reported in patients with CD81 deficiency (79). BCR complex along with toll like receptor mediated signalling is essential for removal of autoreactive B cells. As a result, patients with defect in components of BCR may be predisposed to develop autoimmune cytopenia.

Patients with activated Phosphoinositide 3-kinase (PI3) δ syndrome (APDS) present with a CVID or hyper IgM phenotype with predominant clinical manifestation of autoimmunity (especially autoimmune cytopenia) and lymphoproliferation (80, 81). APDS is caused by a gain of function mutation in the *PIK3CD* gene that encodes for catalytic subunit (p110δ) of PI3Kδ [APDS 1] or loss of function mutations in *PIK3R1* gene that encodes for regulatory subunit (p85α) of PI3Kδ [APDS2] (82). The end result of these molecular defects is an overactivation of the mammalian target of rapamycin (mTOR) pathway that leads to cell survival, cell proliferation and inhibition of apoptosis. Development of autoimmunity in APDS is a complex mechanism. B cell apoptosis in the germinal centre is an important mechanism to eliminate auto-reactive B cells. This mechanism along with B cell hyperactivation and enhanced proliferation may lead to autoimmunity including autoimmune cytopenia (83).

Heterozygous pathogenic variants in the *NFKB2* lead to a CVID phenotype along with a distinct pattern of autoimmune manifestations. Unlike most patients with CVID wherein autoimmune cytopenia is the most common autoimmune manifestations, this is not the most common autoimmune manifestation in patients with NFKB2 gene mutation (84). Autoimmunity in patients with haploinsufficiency of *NFKB2* is more likely to be T cell driven and unlike the mechanism of autoimmunity in other forms of genetic defects causing CVID, there is no significant role of autoantibodies. *NFKB2* also has important role in central tolerance. *NFKB2* signalling is important for the development of medullary thymic epithelial cells and regulation of autoimmune regulator (AIRE). As a result of haploinsufficiency of *NFKB2*, there is loss of central tolerance mechanism leading to accumulation of auto-reactive T cells.

IKAROS a transcription factor in humans encoded by *IKZF1* gene. The somatic mutation in *IKZF1* gene predispose to development of malignancy while more recently patients with germline mutations have been reported to develop immunodeficiency that commonly presents as CVID. Patients with CVID with germ line mutations in *IKZF1* also develop autoimmune cytopenia. IKAROS as a transcription factor controls development of autoimmunity by promoting the B cell anergy and by regulating the TLR pathway signalling (85). It has also been shown that dimerization defective mutations in *IKZF1* gene are more likely to develop autoimmune manifestations as compared to patients with haploinsufficiency mutations. Patients with dominant negative mutations do not develop autoimmunity. The likely mechanism for an increased risk of autoimmune manifestations associated with dimerization defective mutations is an abnormal posttranslational modification of IKAROS and abnormal B cell tolerance (86). **Figure 2** illustrates various genes and downstream pathways involved in pathogenesis of autoimmune cytopenia..

Apart from above mentioned monogenic defects, few more genetic aetiologies have been identified in patients with CVID that may play an important role in the pathogenesis of immune cytopenia in these patients. The field of genetics in patients with CVID is expanding and more than 65 monogenic defects have been identified so far. It is possible that several novel genetic pathways in the pathogenesis of autoimmune cytopenia in patients with CVID would be explored as genetic aetiology of CVID is studied from other populations.

ARE MONOGENIC DEFECTS MORE LIKELY TO BE IDENTIFIED IN PATIENTS WITH CVID WITH AUTOIMMUNE CYTOPENIA?

Monogenic defects may account for up to 50% of all patients depending on the population studied and the techniques used. However, monogenic defects in patients with CVID have only been evaluated in few populations. It has been suggested that patients with CVID from consanguineous families, those who have an affected family member and those with unusual and refractory disease are more likely to have an underlying monogenic defect. However, because of the fact that many of these monogenic defects have an important pathophysiological link with development of autoimmune cytopenia (as discussed above), monogenic defects may possibly be identified more commonly in this subset of patients

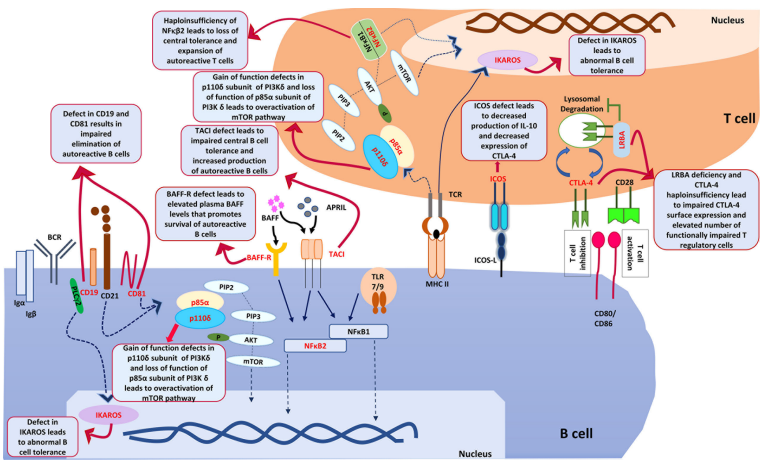

FIGURE 2 | shows various genes and downstream pathways that are involved in pathogenesis of autoimmune cytopenia in patients with CVID. ICOS, Inducible T cell costimulator; ICOS-L, Inducible costimulator ligand; CTLA-4, Cytotoxic T-lymphocyte associated protein 4; LRBA, Lipopolysaccharide responsive beige anchor protein; CD28, Cluster of Differentiation 28; CD80, Cluster of Differentiation 80; CD86, Cluster of Differentiation 86; PIK3Cδ, Phosphatidylinositol (4,5)-bisphosphate 3-kinase δ; PIP2, Phosphatidylinositol (4,5)-bisphosphate; PIP3, Phosphatidylinositol (3,4,5)-trisphosphate; Akt, 'Ak' strain 'thymoma' protein; mTOR, mammalian target of rapamycin; PTEN, PI3K regulatory subunit α; TCR, T cell receptor; MHCII, major histocompatibility complex Class II; NFκB1, Nuclear factor kappa B1; NFκB2, Nuclear factor kappa B2; BCR, B cell Receptor; BAFF, B cell activating factor; BAFF-R, B cell activating factor receptor; APRIL, A proliferation- inducing ligand; PLCγ2, Phospholipase C gamma 2; TACI, Transmembrane activator and calcium modulator and cyclophilin ligand interactor; CD19, Cluster of differentiation 19; CD81, Cluster of differentiation 81; TLR, Toll like receptor; Igα, Immunoglobulin alpha; Igβ, Immunoglobulin beta.

with CVID. In 2 studies that have reported monogenic defects in children with Evans syndrome (autoimmune haemolytic anaemia and thrombocytopenia), more than 2/3rd patients were found to have pathogenic variants in various genes especially in the genes that also predispose to CVID such as LRBA and CTLA-4 (77, 78).

A retrospective study by Ma et al. utilised high-throughput next-generation sequencing (NGS) to identify pathogenic variants in their cohort of children with refractory ITP and it was observed that 9.1% children had pathogenic variants related to CVID {5 *TNFRSF13*; 1 *LRBA*; 1 *NF-κB2*; and 1 caspase recruitment domain 11 (*CARD11*)}. Authors concluded that patients who had recurrent and/or refractory autoimmune cytopenia; propensity to develop recurrent infections and family history of autoimmunity/immunodeficiency need evaluation for underlying monogenic defects (87).

DIAGNOSIS AND MANAGEMENT OF AUTOIMMUNE CYTOPENIA IN CVID

As alluded to previously, autoimmune cytopenia may be the first and only symptom of CVID. This may lead to a diagnostic conundrum as patients with CVID may not produce an adequate autoantibody response and some of the diagnostic laboratory investigations such as direct Coombs' test, anti-platelet antibodies or anti-neutrophil antibodies may give normal results. Thus, it might be prudent to check serum immunoglobulin levels in all patients with unexplained cytopenia (5).

Glucocorticoids have remained the standard of care in autoimmune cytopenia in CVID (88). A review by Cunningham-Rundles reported that most cases of ITP/AIHA respond to oral or intravenous corticosteroids (88). Slow tapering of corticosteroids and immunoglobulin replacement is recommended. Studies have shown recurrence of cytopenia on immunoglobulin replacement therapy, however, the overall frequency as well as morbidity and mortality remain low (3, 88).

In a retrospective multicentre study on 33 patients with CVID-associated refractory immune cytopenias, rituximab showed an initial response rate of 80% and a sustained response rate of 50% at a mean follow-up of 39 months (89).

There are conflicting reports on role of splenectomy in the management of cytopenia in CVID. Initial reports showed an increased rate of mortality following splenectomy (9). However, recent studies have shown if adequate immunoglobulin replacement is being continued, splenectomy has no association with adverse outcomes (**Table 1**). Wong et al. in 2013 reported the outcome of splenectomy in patients with CVID. Splenectomy was found to be an effective long-term treatment in 75% patients with CVID with autoimmune cytopenia, even in those who were non-responsive to rituximab. Splenectomy did not increase the risk of mortality and appropriate replacement immunoglobulin therapy appeared to be sufficient for prevention of overwhelming post-splenectomy infections (90).

Current guidelines on chronic ITP and aplastic anaemia recommend the usage of thrombopoietin-receptor agonists (TPO-A) as a second or third-line agent in refractory cases (91). In CVID associated ITP, authors have suggested use of TPO-A as an alternative to splenectomy and rituximab in refractory cases (92).

Monogenic forms of CVID are often associated with autoimmune cytopenia (6). Although conventional immunosuppression and immunomodulation such as corticosteroids may work in the presence of monogenic defects, targeted therapies are now being used in these disorders (93, 94).

The mTOR pathway has been reported to be activated inn patients with APDS and mTOR inhibitor, sirolimus has been recommended for management of cytopenia and lymphoproliferation (93). However, Maccari et al. reported that sirolimus was not as effective in the management of cytopenia as it is for lymphoproliferation in these patients (95). Recently, selective PI3Kδ inhibitors such as leniolisib have also been tried (93).

Sirolimus has also been used in management of cytopenia in other monogenic forms of CVID such as LRBA deficiency and CTLA-4 haploinsufficiency with variable results. Abatacept, a CTLA-4 immunoglobulin fusion drug has been found to be an effective treatment modality for autoimmune cytopenia in these disorders. A long-term outcome study by Tesch et al. reported that disease activity scores were significantly lower in patients who were on abatacept therapy as compared to other forms of therapies (94). A recent large study on patients with CTLA-4 haploinsufficiency, cytopenia was managed using corticosteroids, rituximab, abatacept, splenectomy and immunomodulatory doses of IVIg (96). Sirolimus was not used for management of cytopenia. Following corticosteroids (that showed transient response in most patients), rituximab was the most commonly used drug and showed good efficacy in the management of cytopenia. Splenectomy produced a sustained response in 1/4th of cases where it was carried out. It was also suggested that immunoglobulin replacement therapy does not prevent or ameliorate disease related complications in patients with CTLA-4 haploinsufficiency. Hematopoietic stem cell transplantation (HSCT) is an effective option for refractory cytopenia. Autoimmune cytopenia in patients with LRBA deficiency have also been managed on similar lines as in patients with CTLA-4 haploinsufficiency. However, rituximab has been used less commonly while abatacept has been used more commonly in the former group (97).

With identification of more genetic defects in patients with CVID in future, more targeted therapies are likely to be explored for management of various disease related complications such as autoimmune cytopenia.

CONCLUSIONS

Autoimmune cytopenia is the most common autoimmune manifestation in patients with CVID. Patients with CVID with autoimmune cytopenia have unique immunophenotypic abnormalities in the B and T cell compartment. The pathophysiology of autoimmune cytopenia in CVID has been linked to an abnormality in both B and T cell compartment as well in the innate arm of the immune system. Patients with CVID with autoimmune cytopenia (especially in patients who have Evan's syndrome) are more likely to have an underlying

monogenic defect. The treatment of choice for autoimmune cytopenia in CVID remains corticosteroids. However, biologic drugs and several targeted treatments are now being explored.

AUTHOR CONTRIBUTIONS

SC- Preparation of the first draft of the manuscript; editing of manuscript; literature review. PB- Preparation of the first draft of the manuscript; editing of manuscript; literature review. RT- Preparation of the first draft of the manuscript; editing of manuscript; literature review. AJ- Preparation of the first draft of the manuscript; editing of manuscript; literature review; critical review and final approval. SSh/AR- Editing of manuscript, review of literature. SSi- Critical revision of manuscript, review of literature and final approval. All authors contributed to the article and approved the submitted version.

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Features of Hemophagocytic Lymphohistiocytosis in Infants With Severe Combined Immunodeficiency: Our Experience From Chandigarh, North India

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Background: Hemophagocytic lymphohistiocytosis (HLH) is characterized by uncontrolled and excessive inflammation leading to high mortality. Aetiology of HLH can be primarily due to genetic causes or secondarily due to infections or rheumatological illness. However, rarely T-cell deficiencies like severe combined immunodeficiency (SCID) can develop HLH.

Objective: To describe clinical and laboratory features of SCID cases who developed HLH.

Methods: We collected clinical, laboratory, and molecular details of patients with SCID who developed HLH at our center at Chandigarh, North India.

Results: Of the 94 cases with SCID, 6 were noted to have developed HLH-like manifestations. Male-female ratio was 5:1. Median (inter-quartile range) age of onset of clinical symptoms was 4.25 months (2-5 months). Median (inter-quartile range) delay in diagnosis was 1 month (1-3.5 months). Family history of deaths was seen in 4 cases. Molecular defects in *IL2RG* were seen in 5 out of 6 cases. Documented infections include disseminated bacillus calmette-guerin (BCG) infection (n=2), blood stream infections (n=3) with *Staphylococcal aureus* (n=1), *Klebsiella pneumonia* (n=1), and *Pseudomonas aeruginosa* (n=1), pneumonia (influenza H1N1 strain, and K. *pneumoniae* (n=1).

Conclusion: Children with SCID can present with HLH-like manifestations secondary to fulminant infections. A high index of suspicion of SCID is needed in infants who present with HLH who have an associated infection or a suggestive family history. Occurrence of HLH-like manifestations in SCID suggests that T-lymphocytes may not have a significant role in immunopathogenesis of HLH.

Keywords: severe combined immunodeficiency, hemophagocytic lymphohistiocytosis, infections, BCG, family history, X-linked

1 INTRODUCTION

Severe combined immune deficiency (SCID) is a heterogeneous group of disorders caused by a variety of genetic abnormalities (1) (2). It is characterized by defective T and B lymphocyte function leading to life threatening infections and mortality if not treated with HSCT on time.

Hemophagocytic lymphohisticytosis (HLH) is a life-threatening condition due to immune dysregulation characterized by multi-organ dysfunction, rapidly progressive cytopenias, hypertriglyceridemia, hypofibrinogenemia, and hyperferritinemia. Genetic causes of primary HLH include *PRF1, STX11, STXBP2, MUNC, UNC13D, RAB27A, LYST, AP3B, SH2D1A*, and *BIRC4* defects or other primary immunodeficiency diseases such as SCID and chronic granulomatous disease (3) (4).

Diagnosis of SCID in an infant presenting with HLH-like manifestations can be a challenge for clinical immunologists in view of symptoms masquerading as sepsis and multiorgan dysfunction. Also, the rapidly progressive bicytopenia/pancytopenia makes interpretation of lymphocyte subsets by flow cytometry a challenging task. Management of HLH-like manifestations in an infant with SCID is equally challenging because almost always, an infection would be the trigger, and treating the infection becomes essential. We report our cohort of 6 patients with SCID from Northern India who developed HLH-like manifestations and provide a brief review of literature. To the best of our knowledge, reports of HLH in SCID from developing nations are not available.

2 METHODS

Medical records of children diagnosed with SCID at the Allergy and Immunology Unit, Advanced Pediatrics Centre, Post Graduate Institute of Medical Education and Research over the last 2 decades were retrieved and analyzed. Clinical data included demographic details, family history clinical examination findings, and pattern of infection, number of infections, type of infections, site of infections, organism involved, age of presentation, age of onset, presence of skin rash, and BCG ulceration. Hematological parameters included complete blood count, coagulation profile, serum fibrinogen levels, and bone marrow examination findings. Biochemical investigations including liver enzymes, ferritin, renal functions, lipid profile, and C-reactive protein were also analyzed.

Diagnosis of SCID was based on laboratory or genetic documentation. Diagnosis of HLH was made on the basis of HLH 2004 criteria (5). Analysis of lymphocyte subsets by flow cytometry was carried out in all patients. Laboratory assay of lymphocyte subsets, naïve, memory T cells, HLA-DR expression, CD132 expression, and lymphocyte proliferation assays were carried out as previously described (6). Immunoglobulin levels were estimated by nephelometery.

2.1 Lymphocyte Subset Analysis By Flow Cytometry

A total of 50 µL of EDTA blood sample is mixed with 4 µL of antibody mixture (CD45 ECD-*Beckman Coulter*), B cells (CD19 FITC- *Beckman Coulter*), T lymphocytes (CD3 PE CY7-*Beckman Coulter*), and natural killer cells (CD56 APC-*Beckman Coulter*). The mixture is vortexed and then incubated in the dark for 20-30 min at room temperature. A total of 1 ml RBC lysis buffer was then added and incubated for 15 min at room temperature. Centrifugation at 1500 rpm for 5 min was done and supernatant was decanted. There was 1 ml sheath fluid added to the pellet for washing and the tubes are centrifuged again at 1500 rpm for 5 min. The pellet was then resuspended in 300-500 µL sheath fluid. A sample was then acquired on the Beckman Coulter™ *Navios* flow cytometer. Lymphocytes were first gated using SSc vs. CD45 and different subsets were then estimated on gated lymphocytes. Analysis was done using *Kaluza* software.

2.2 Surface CD132 Expression By Flow Cytometry

There was 50 µL of EDTA blood sample mixed with 4 µL of antibody – CD132 PE (*Becton Dickinson*). The mixture is vortexed and then incubated at room temperature in the dark for 20-30 min. A total of 1 ml RBC lysis buffer was then added and incubated for 15 min at room temperature. Centrifugation at 1500 rpm for 5 min was done and supernatant was decanted. There was 1 ml sheath fluid added to the pellet for washing and the tubes are centrifuged again at 1500 rpm for 5 min. The pellet is now resuspended in 300-500 µL sheath fluid. A sample was then acquired on the Beckman Coulter™ *Navios* flow cytometer.

Lymphocytes, monocytes, and neutrophils were gated from the FS vs. SS plots and surface expression of common γ chain (CD132) on lymphocytes, monocytes, and neutrophils was done and compared with healthy controls. Analysis was done using *Kaluza* software.

2.2.1 Molecular Analysis

Molecular analysis for patients (P2, P3) was performed at our institute. Molecular diagnosis for 2 patients (P5, P6) was established at Kazusa DNA Research Institute, Japan and National Defense Medical College, Saitama and Tokyo Medical and Dental University, Tokyo, Japan. Molecular tests for 2 patients (whole-exome sequencing) were carried out from a private laboratory in India (P1, 4). Next-generation sequencing (Ion Torrent, Thermo Fisher Scientific India Pvt. Ltd.) for clinical care was started in July 2018 at the Advanced Pediatrics Centre, PGIMER, Chandigarh. A targeted PID gene panel comprising 44 genes was used that covered 7 genes for SCID – *ADA*, *RAG1*, *RAG2*, *IL2RG*, *JAK3*, *IL7RA*, and *LIG4.* Antenatal diagnosis was performed for 3 families of these patients.

2.3 Search Strategy

We searched Pubmed, MEDLINE, Embase, and Scopus databases for published literature using the following search term on December, 2021: severe combined immunodeficiency and hemophagocytic lymphohistiocytosis. A total of 53 articles were reviewed and studies and case reports and series which showed development of HLH in a SCID patient were selected and reviewed (**Table 5**)

3 RESULTS

Over the last 20 years, we have diagnosed 94 children with SCID at our center (7). Six children were noted to have developed HLH-like manifestations and 4 patients fulfilled the HLH-2004 criteria for diagnosis of HLH. The remaining 2 patients were considered to have a probable HLH. In these 6 patients, the male-female ratio was 5:1. Median (inter-quartile range) age of onset of clinical symptoms of SCID (onset of first documented infection) was 4.25 months (2-5 months). Median (inter-quartile range) delay in diagnosis of SCID was 1 month (1-3.5 months). Family history of deaths was seen in 4 cases. Molecular defects in *IL2RG* were seen in 5 out of 6 cases. However, final genetic diagnosis is not available in one patient (P3) as NGS for targeted PID panel performed at our center has not yielded any defect. Documented infections include disseminated bacillus calmette-guerin (BCG) infection (n=2), blood stream infections (n=3) with *Staphylococcal aureus* (n=1), *Klebsiella pneumonia* (n=1), and *Pseudomonas aeruginosa* (n=1), pneumonia (influenza H1N1 strain and K. *pneumoniae* (n=1)).

Features of HLH were noted at the time of presentation in 2 (P1, P3) children and during the hospital stay in the rest. Fever and splenomegaly were noted in 6 (100%) and 5 cases (83.3%), respectively. Laboratory features of cytopenia, hyperferritinemia, hypertriglyceridemia, and hypofibrinogenemia were seen in 6 (100%), 4 (66.6%), 2 (33.3%), and 4 (66.6%) cases, respectively (**Table 1**). While bone marrow evidence of HLH was documented in 3 cases, post-mortem histopathological evidence was seen in 2 cases.

3.1 Patient 1

A 6-month-old boy, second born to a non-consanguineously married couple presented with high grade fever for 1 month. Fever was associated with cough and rapid breathing that was non-paroxysmal with no postural or diurnal variations for 15 days. He also developed watery loose stools associated with excessive perianal rash and excoriation. Parents also gave history of recurrent oral thrush. For these symptoms, child was treated elsewhere with intravenous antimicrobials and referred to us in view of no improvement. His elder male sibling expired at 4 months of age with pneumonia and diarrhea (**Supplementary Figure 1**). On examination, he had pallor, oral thrush, tachypnea, and tachycardia with intercostal retractions. Abdominal examination revealed splenomegaly (4 cm below left costal margin) and hepatomegaly (palpable 4 cm below right costal margin). Blood investigation showed pancytopenia with absolute lymphocyte count (ALC) 0.128 x 10^9/L. Liver function tests showed elevated aspartate transferase (AST) 283 IU/L (N=15-40 IU/L) and alanine transferase (ALT) 49 IU/L (N=12-45 IU/L). In view of pancytopenia, persistent fever, and hepatosplenomegaly, HLH work up was sent that revealed hyperferritnemia, hypertriglyceridemia, and hypofibrinogenemia (**Table 1**). HIV serology was non-reactive. Possibility of primary HLH vs. SCID was considered. Flow cytometry was suggestive of extremely low proportions of T lymphocytes and natural killer cells (**Table 2**). Blood culture has shown growth of *K. pneumonia*. Chest x ray (CXR) showed bilateral infiltrates and thymus shadow was absent. Gastric lavage (GL) for acid-fast bacilli (AFB) staining, cartridge-based nucleic acid amplification test (CBNAAT) for *Mycobacterial tuberculosis* and smear for *Pneumocystis jirovecii* yielded negative results. Qualitative PCR for cytomegalovirus from peripheral blood was negative. The child was treated with broad spectrum antimicrobials, IV cotrimoxazole, IV Amphotericin B, and oral 4-drug antitubercular therapy (ATT). In view of HLH, intravenous immunoglobulin (IVIg) was given at 2 gm/kg. However, the pneumonia worsened requiring mechanical ventilation and he succumbed to the illness. Genetic analysis revealed *IL2RG* defect (**Supplementary Figure 1**). Antenatal diagnosis was offered for

TABLE 1 | Clinical and laboratory features of HLH in patients with SCID from our cohort.

Investigations /clinical	P1	P2	P3	P4	P5	P6
Fever	Yes	Yes	Yes	Yes	Yes	Yes
Splenomegaly	Yes	Yes	No	Yes	Yes	Yes
Hemoglobin (g/L)	7.1	4.6	6.5	9.8	6.2	5
Total leucocyte count (× 10^9 /L) (N: 4-11)	2.14 ↓	2.34 ↓	35.4 ↑	4.17	15	18.8 ↑
Differential leucocyte counts (N%/L% /M%/E%)	85/6/8/0	81/16/2/1	92/6/0/2	85/9/5/0	69/25/6/0	90/5/3/2
Platelet counts (×10^9 /L) (N: 150-450 ×10^9 /L)	21 ↓	4 ↓	16 ↓	150 ↓	93 ↓	14 ↓
CRP (mg/L) (N: <10)	90	204	144	78.58	NA	NA
ESR (mm/1st hour) (N:0-10)	NA	2	NA	NA	NA	NA
Ferritin (ng/ml) (N:30-400)	3365 ↑	967 ↑ 1703	36016 ↑	>2000 ↑	61900 ↑	NA
Fibrinogen (g/L) (N:2.5-5)	<0.4 ↓	2.12	<0.35 ↓	0.46 ↓	1.08 ↓	NA
Triglyceride (mg/dl) (N: <100)	217 ↑	NA	172 ↑	255 ↑	>500 ↑	NA
Aspartate Transferase (IU/L) (N:15-50)	283 ↑	246 ↑	2988 ↑	133 ↑	2280 ↑	604 ↑
Alanine Transferase (IU/L) (N:12-45)	49	72 ↑	378 ↑	116 ↑	609 ↑	50
Bone marrow	NA	hemophagocytosis	NA	hemophagocytosis	hemophagocytosis	Erythrophagocytosis
Soluble CD25	NA	89.47 IU/ml (27-189 IU/ml)	NA	NA	NA	NA
HLH 2004 Criteria	Yes	Yes	No	Yes	Yes	No
HLH diagnosed on	Day 1	Day 7 HS	Day 1	Day 9	D5 HS	Post mortem/Autopsy

P, patient; N, neutrophils; L, lymphocytes; M, monocytes, E, eosinophils; HLH, hemophagocytic lymphohistiocytosis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HS, hospital stay, High Low. NA, Not available.

TABLE 2 | Immunological work-up of patients with SCID and HLH-like manifestations at the time of SCID diagnosis.

Immunological work up	P1	P2	P3	P4	P5	P6
Hemoglobin(g/L)	7.1	5.3	6.4	9.8	6.2	5
Total leucocyte count (× 10^9 /L) (N: 4-11)	2.14 ↑	14.9 ↓	9.5	4.17	22.6 ↑	18. 8 ↑
Differential leucocyte counts (N%/L%/M%/E%)	85/6/9/0	85/14/0.9 /0.1	92/6/0/2	85/9/4/2	87/8/3/2	90/5/ 3/2
Platelet counts (× 10^9 /L) (N: 150-450 ×10^9 /L)	21 ↓	95 ↓	390	150	276	14 ↑
Absolute lymphocyte count (× 10^9 /L)	0.128	2.086	0.285	0.375	1.8	0.940
%CD3 T lymphocytes Absolute counts	2.46% (49-76% 03 (1900-5900) ↓	0.61% (51-77%) 13(2500-5600) ↓	82.87% (53-84%) 236 (2500-5600) ↓	1.7% (15-77%) 6.4 (2500-5600) ↓	95.65% (49-76%) 1721 (2500-5600) ↓	Absent
%CD 19 B lymphocytes Absolute counts	87% (14-37%) 111 (610-2600)	97.82% (11-41%) 2041 (430-3000)	5.69% (6-32%) 16 (430-3000) ↓	91.57% (11-41%) 343 (430-3000)	1.78% (14-37%) 32 (430-3000) ↓	86% (14-37%) 808 (430-3000)
%CD 56+ NK lymphocytes Absolute counts	2.51% (3-15%) 3 (160-950) ↓	0.22% (03-14%) 5 (170-830) ↓	5.63% (4-18%) 16 (170-830) ↓	2.68% (3-14%) 10 (170-830) ↓	0.53% (3-15%) 10 (170-830) ↓	NA NA
%CD3+ CD 56+ NK lymphocytes	0.64%	0.04%	2.07%	0.05%	1.69%	NA
%CD 45 RO+ of CD3 +lymphocytes	–	–	89.37% (control 45.92%)	81.8% (control 24.58%)	97.91%	–
%CD 45 RA+ of CD3 +lymphocytes	–	–	12.07% (control: 54.30%)	00.16% (control: 46.29%)	0.34%	–
%HLA DR+ on CD3 +lymphocytes	–	–	Mild increase (15.1%) as compared to healthy control (10.18%)	–	Increased (82.15%) as compared to control (49%)	–
CD 132	Decreased on lymphocytes (12.07%) as compared to control (40.53%)	Decreased on lymphocytes 25.23% as compared to control (83.53%)	–	–	Decreased on lymphocytes (67.8% vs 88.3%) and monocytes (59.7% vs 88.9%)	–
IgG(g/L) IgA(g/L) IgM(g/L)	–	IgG: <1.46 (2.40-8.80) IgM: 0.17 (0.20-10.0) IgA: 0.24 (0.10- 0.50)	–	IgG 0.232 Ig A <0.20 IgM 0.21	IgG <0.202 IgA 0.20 IgM 0.171	IgG <0. 294 IgA <0.49 IgM <0.88
VNTR	NA	NA	NA	NA	No maternal engraftment	NA
Type of SCID	IL2RG	*IL2RG*	*NA*	*IL2RG*	*1L2RG*	*IL2RG*

P, patient; N, neutrophils; L, lymphocytes; M, monocytes, E, eosinophils; VNTR, variable number of tandem repeats. NA, Not available.

the subsequent pregnancy for parents, and the fetus was found to be unaffected.

3.2 Patient 2

A 5-month-old boy, first born to a non-consanguineously married couple, presented with high grade fever and rash for 1 month. Rash was maculopapular, non-blanchable over the entire body which healed with hyperpigmented papules. He also had cough and watery loose stools. For these symptoms, the child was treated elsewhere with intravenous antimicrobials and referred to us in view of no improvement. There was no significant family history. On examination, he had pallor, oral ulcers, palpable

papules, and hyperpigmented rash all over the body. The BCG site was ulcerated with minimal pus discharge. Tachypnea and tachycardia with intercostal retractions were also noted. Abdominal examination revealed splenomegaly (9 cm below left costal margin) and hepatomegaly (palpable 5 cm below right costal margin). Blood investigation showed anemia and lymphopenia (**Table 2**). Liver function tests showed elevated liver enzymes. HIV serology was non-reactive. Possibility of SCID was considered with BCGosis in view of ulceration and pus discharge at BCG vaccination site. Flow cytometry was suggestive of extremely low proportions of T lymphocytes and natural killer cells (**Table 2**). CXR and ultrasound (USG) confirmed the absence of the thymus. USG abdomen revealed multiple tiny hypoechoic lesions in the liver and spleen. Biopsy from skin nodules showed acid-fast bacilli (**Figure 1**). Pus from the BCG site and stool have also shown AFB smear positivity, and CBNAAT positive (rifampicin sensitive) for *M.* tuberculosis complex. Hence, a diagnosis of disseminated BCGosis (lung, skin, gut, liver, spleen) with SCID was made and was started on 4-drug ATT. Infective work-up including blood cultures, and PCR for cytomegalovirus was negative. He was also given broad spectrum anti-microbials, IV Cotrimoxazole and IV Amphotericin B. In view of persistent fever despite therapy, HLH was considered. Work up was suggestive of hyperferritinemia and hypofibrinogenemia with progressive fall in platelet count and leukocyte count (**Table 1**). His soluble CD25 level was high 89.47 U/ml (27-189 U/ml). IVIg was given at 1 gm/kg. However, his pneumonia worsened, and he succumbed to the illness. Genetic analysis revealed *IL2RG* defect (**Tables 3**, **4**). Post-mortem bone marrow examination showed evidence of hemophagocytosis (**Figure 1**). Antenatal diagnosis was offered for the subsequent pregnancy for parents, and the fetus was found to be unaffected.

3.3 Patient 3

A 3-month-old girl, unvaccinated baby, sixth born to a non-consanguineously married couple, presented with high grade fever and rash for 1 month. She developed vesicular lesions on the trunk which progressively involved the whole body which later discharged pus. Three days prior to presentation to our institute, she developed rapid breathing and watery loose stools associated with abdominal distension. For these symptoms, the child was treated elsewhere with intravenous antimicrobials and blood transfusion was given and she developed diffuse redness of the body post transfusion and diarrhea. There was a significant family history with three elder sibling deaths (**Supplementary Figure 2**). On examination, she had pallor, anasarca, and bullous pus-filled pustular lesions and erythroderma. She had tachypnea and tachycardia with intercostal retractions and bilateral crepitations. Abdominal examination revealed hepatomegaly (liver 3 cm below right costal margin). Blood investigation done elsewhere showed anemia with absolute lymphocyte

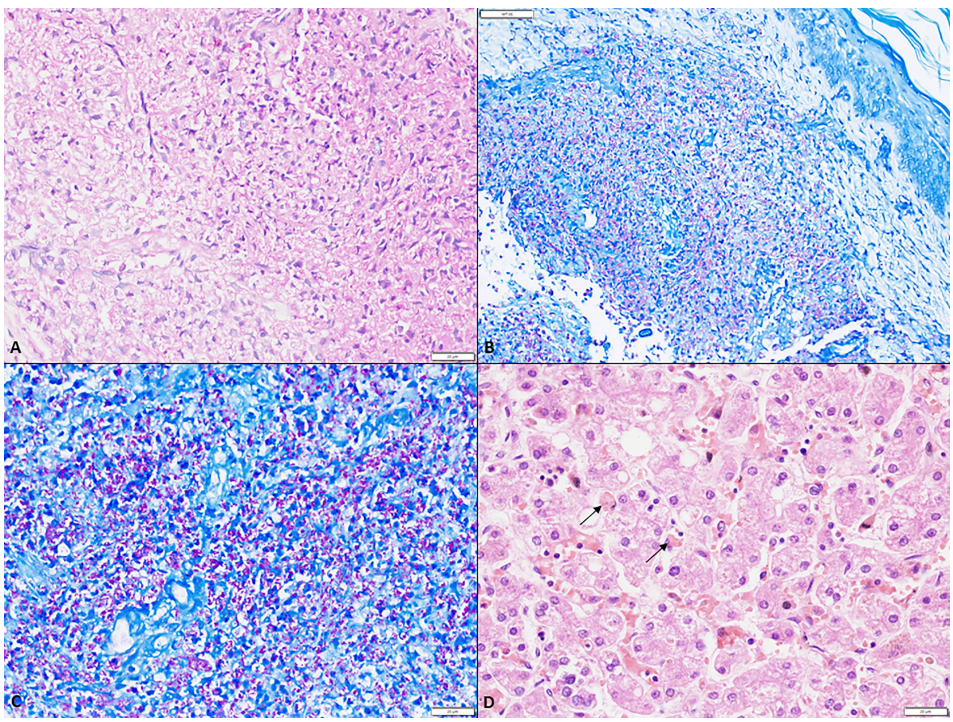

FIGURE 1 | **(A)** A large collection of foamy macrophages in the dermis (scale bar 20 μm); **(B)** low magnification depicting numerous acid-fast bacilli within the foamy cells in the dermis (ZN, scale bar 50 μm); **(C)** Numerous acid fast bacilli within the dermis both intra- and extra-cellularly (ZN, scale bar 20 μm); **(D)** Hemophagocytosis within Kupffer cells in the sinusoids (arrow) (H&E, scale bar 20 μm).

TABLE 3 | Clinical features of patients with HLH-like features in SCID in our series.

Parameters	P1	P2	P3	P4	P5	P6
Family history	Yes	No	Yes	No	Yes	Yes
Age of onset (months)	5	4	2	5	4.5	3
Age of diagnosis (months)	6	5	3	6	6	6.5
Delay of diagnosis (months)	1	1	1	1	1.5	3.5
HLH (months)	6	5	3	6	6	6.5
BCG site	No scar	Ulcerated	No scar	Normal scar	Normal scar	No scar
History of blood transfusions	No	No	Yes	Yes	Yes	No
Rash	No	Yes (BCG)	Yes (post-transfusion)	Yes	Yes	No
Omenn/GVHD/maternal engraftment	No	No	GVHD (secondary to blood transfusion)	Omenn syndrome	Omenn syndrome	No
Infection	Pneumonia Diarrhea	Pneumonia	Pneumonia Diarrhea	Pneumonia	Pneumonia Diarrhea	Recurrent pneumonias
Organism isolated by microbiological methods	Blood culture: *Kleibsiella pneumonia*	Disseminated BCG infection	Blood culture staphylococcus aureus	H1N1; K. pneumoniae (oropharyngeal)	No organism	Disseminated BCG in thymus, lungs, lymph nodes, spleen, liver, kidney, bone marrow
Treatment regimen/ Immunomodulation	IVIg 1g/kg IV antimicrobials, antifungals, ATT	IVIg 1g/kg IV antimicrobials, antifungals, ATT	IVIg 2 g/kg IV antimicrobials, antifungals	IVIg IV antimicrobials, antifungals, ATT	IVIg IV antimicrobials antifungals	IV antimicrobials IVIg 1 g/kg

count 0.285 x 10^9/L. When she was investigated in our institute, we noted anemia, thrombocytopenia, and elevated liver enzymes. Blood culture has shown growth of *Staphylococcal aureus*. Qualitative PCR studies for cytomegalovirus and Epstein-Barr virus (EBV) from peripheral blood yielded negative results. A possibility of SCID with graft vs. host disease (GVHD) post transfusion was considered along with HLH. HLH work up was sent which revealed hyperferritnemia, hypertriglyceridemia, and hypofibrinogenemia (**Table 1**). Flow cytometry was suggestive of SCID (**Table 2**). IVIg was given at 2 gm/kg along with IV antimicrobials including cotrimoxazole and amphotericin B. She had further respiratory worsening, refractory shock, and succumbed to the illness. A targeted PID gene panel comprising 44 genes was used that covered 6 genes for SCID – *ADA*, *RAG1*, *RAG2*, *IL2RG*, *JAK3*, *IL7RA*, and *LIG4* and HLH genes *PRF1 and STX11* but did not yield any defect among these genes.

3.4 Patient 4

A 6-month-old boy first born to a non-consanguineously married couple presented with high grade fever and cough for 1 month. He developed rapid breathing for 15 days prior to admission. Influenza *H1N1* strain and *K. pneumonia* were isolated from nasopharyngeal secretions. He was referred to us in view of no improvement. In the past, he had watery loose stools at 1 week of age requiring hospitalization. There was no significant family history. On examination, he had pallor, tachypnea, and tachycardia with intercostal retractions and bilateral crepitations. Abdominal examination revealed splenomegaly (1 cm below left costal margin) and hepatomegaly (palpable 3 cm below right costal margin). Blood investigation showed anemia and lymphopenia. He was ventilated for progressive respiratory worsening. Work-up for *M. tuberculosis* complex and *P. jirovecii* were negative. He was

TABLE 4 | Detailed genetic reports of SCID patients with HLH in the present series.

Patient	Gene	Exon	Protein position	c.DNA	Inheritance pattern	Type	Novelty	Pathogenicity
P1	IL2RG	Intron 7		c.924+1 G>A	X-Linked	Splice –site (Hemizygous)	Yes	Pathogenic
P2	IL2RG	5	p.E199VfsX76	c.596_598delinsTGGATTAT	X-Linked	Hemizygous –Frameshift Indel (InsTGGATTAT delAAC)	Previously reported **(7)**	**Pathogenic**
P3	NA							
P4	IL2RG	8	p.Q322X	c.964C>T	X-Linked	Nonsense (Hemizygous)	Previously reported (7)	Pathogenic
P5	IL2RG	4	p.V152A	c.455 T>C	X-Linked	Missense (Hemizygous)	Previously reported (7)	Pathogenic
P6	IL2RG	4	p.L172R	c.515T>G	X-Linked	Missense (Hemizygous)	Previously reported (7)	Pathogenic

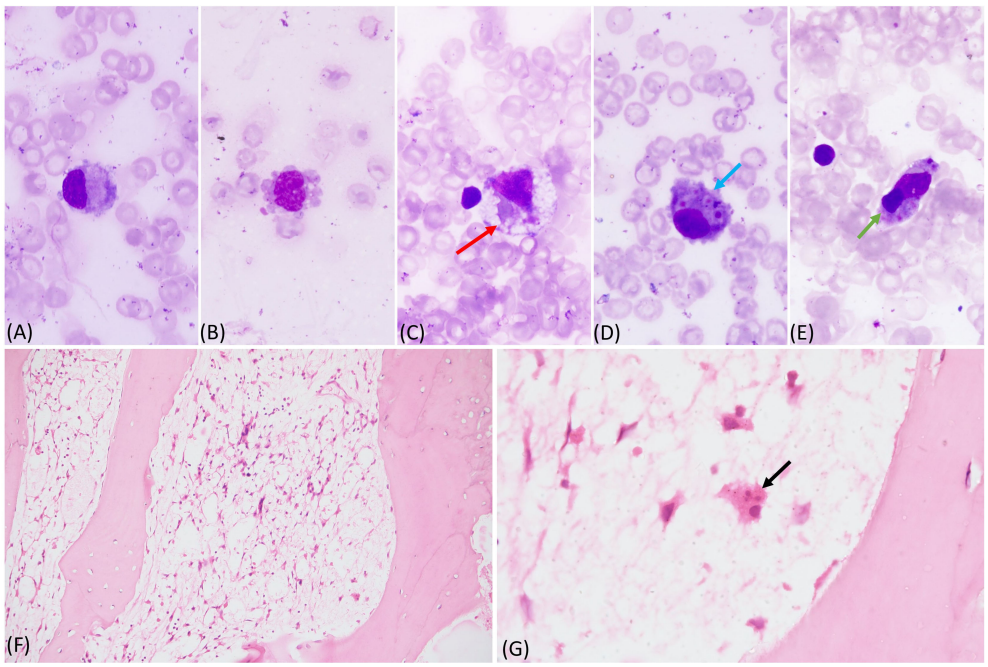

FIGURE 2 | **(A-E)** Bone marrow aspirate showing histiocytes with pseudopods and vacoules, phagocytosed red cells (red arrow), platelets (blue arrow), and lymphocyte (green arrow) (May Grunwald Giemsa stain 100x); **(F, G)** markedly hypocellular bone marrow biopsy with marked reduction of all normal hematopoietic cells. A histocyte is visible with phagocytosed neutrophil (black arrow) (Hematoxylin and Eosin, F - 20x and G - 100x).

treated with IV oseltamivir, and broad-spectrum antibiotics and antifungals. By Day 7 of the hospital stay, there was fall in hemoglobin, leukocyte count, and platelets, requiring blood transfusions. At this point HLH was considered and work-up revealed hyperferritnemia, hypertriglyceridemia, and hypofibrinogenemia (**Table 1**). Bone marrow was suggestive of hemophagocytosis (**Figure 2**). He was given IVIg and dexamethasone. By Day 12 of the hospital stay, he developed new onset erythematous macular rash on the cheeks that progressed to involve the whole body. A probable Omenn syndrome (OS) was considered in view of the development of lymphocytosis and eosinophilia. Liver function tests showed elevated liver enzymes. Flow cytometry was suggestive of extremely low proportions of T lymphocytes and natural killer cells (**Table 2**). Qualitative PCR for cytomegalovirus from peripheral blood was negative. Clinical symptoms further worsened, and he succumbed to illness. Genetic analysis revealed *IL2RG* defect (**Table 4**).

3.5 Patient 5

A 6-month-old boy developmentally normal, immunized for age, first born to a non-consanguineously married couple presented with high-grade fever and cough for 1.5 months. Cough was progressively increasing in severity and was associated with rapid breathing for 15 days prior to presentation. He also developed watery diarrhea for 15 days. For these symptoms, the child was treated elsewhere with intravenous antimicrobials. He also received a blood transfusion because of anemia. In view of worsening respiratory distress, he was referred to our center. There was a significant family history with deaths of 5 maternal uncles by the age of 6 months due to respiratory illnesses (**Supplementary Figure 1**). On examination, he had pallor, generalized macular rash all over the body, and a healthy BCG scar. Chest examination revealed bilateral crepitations. Abdominal examination revealed splenomegaly (4 cm below left costal margin) and hepatomegaly (palpable 5 cm below right costal margin). Blood investigations showed lymphopenia (absolute lymphocyte count 1.808 x 10^9/L) and elevated liver enzymes. CXR and computed tomography of chest revealed diffuse bilateral consolidations. The child was initiated on broad spectrum IV antimicrobials. HIV serology was non-reactive. A possibility of SCID was considered with the presence of family history and lymphopenia. Flow cytometry showed low T cells, decreased naïve T cells, expanded HLA-DR, and decreased CD132 expression (**Table 2**). By Day 5 of the hospital stay, HLH was considered in view of persistent fever, transaminases, anemia, and fall in platelet count to 93x10^9/L and work-up revealed hyperferritnemia, hypertriglyceridemia, and hypofibrinogenemia (**Table 1**). Infective work-up including blood cultures and PCR for cytomegalovirus was negative. A variable number of tandem repeats (VNTR) analysis was done and there was no evidence of maternal engraftment. In view of HLH, IVIg was given at 1 gm/kg and was planned for steroids but the child succumbed to the illness. Genetic analysis revealed *IL2RG* defect (**Table 4**).

3.6 Patient 6

A 6.5-month-old boy, sixth born to a non-consanguineously married couple presented with cough for 2 months. Cough progressively increased in severity and was associated with rapid breathing and fever for 15 days prior to presentation. Parents also noted abnormal body movements of left limbs with altered sensorium. The child had significant history in the form of recurrent pneumonia since the age of 3 months requiring hospitalizations. There was significant family history with the deaths of 3 male elder siblings at the ages of 2, 5, and 6 months, respectively (**Supplementary Figure 1**). On examination, there was failure to thrive and pallor. Chest examination revealed intercostal retractions and bilateral crepitations. Abdominal examination revealed splenomegaly (4 cm below left costal margin) and hepatomegaly (palpable 5 cm below right costal margin). Blood counts showed progressive pancytopenia. Blood culture showed growth *Pseudomonas aeruginosa*. CXR showed diffuse perihilar infiltrates. Work-up for cytomegalovirus and EBV was negative. A possibility of SCID was considered in the presence of family history and lymphopenia. Flow cytometry showed absent T lymphocytes (**Table 2**). He was treated with antimicrobials and IVIg 1 gm/kg for SCID, however, he succumbed to illness by Day 9 of stay. An autopsy was performed that showed thymic atrophy, marked lymphoid depletion, disseminated *BCG* in the thymus, lungs, lymph nodes, spleen, liver, kidney, and bone marrow, and bronchopulmonary aspergillosis and erythrophagocytosis in bone marrow and lymph nodes. Genetic analysis revealed *IL2RG* defect (**Table 4**). Antenatal diagnosis was offered for the subsequent pregnancy for parents, and the fetus was found to be unaffected.

4 DISCUSSION

Ours is the first study of HLH-like manifestations in children with SCID from the Asia Pacific region. The frequency of this rare, yet life-threatening manifestation in our series is 6.38% (6/94) with pathogenic mutations in *IL2RG* in 5 out of 6 cases. Infective triggers have been documented in 5 cases and GVHD/Omenn phenotype was noted in 3 cases.

The first case report of HLH in SCID was reported in 2000 by Grunebaum et al. (8) in a 9-week-old child with X-linked SCID. This was a presenting manifestation of SCID and there were no infections identified at onset. Subsequently, several other case reports have been published (9–18) (**Table 5**). Later, Bode et al. (3) described a larger cohort of cases (n=63) with PID who developed HLH. In this study, 12 patients had SCID and 18 had partial T-cell deficiencies. The most common mutation in these SCID was that of *IL2RG* (n=5), followed by *RAG1* (n=2). In another study by Cetinkaya et al., 4 had SCID, of which mutations in the *RAG1* were identified in 2 patients (4). With the available literature, the most common types of SCID to develop HLH or HLH-like manifestations are X-linked SCID followed by RAG defects. However, features of HLH have also been described with *JAK3*, *CD3D*, *ADA*, and *ORAI1* defects also (13) (15) (16) (18), (20). In India, autosomal forms of SCID are more common than X-linked SCID. However, we observed that the most common type of SCID associated with HLH was *IL2RG* defect (X-linked SCID). Hence HLH in an infant, especially less than 6 months with an X-linked family history should guide us to investigate for SCID, as almost all primary HLH that have been described to date are autosomal recessive in nature. Increased incidence of HLH in *IL2RG* defect is probably due to the defective natural killer function. We document a wide range of mutations – missense, splice-site, and frameshift defects in *IL2RG* in our patients who have developed HLH. Therefore, it appears that type of mutation has no influence on the development of HLH in X-linked SCID.

Most of the time, HLH in PIDs/SCID is triggered by infections. Attempts to isolate an organism becomes important in the management of HLH. Management of secondary HLH can be challenging especially in cases of SCID because the presence of severe infections may hinder the use of aggressive immunosuppression. In the series by Bode et al. (3), 50/63 PID patients (79%) with HLH syndrome had associated infections. In 12 children with SCID, the most common organisms isolated were cytomegalovirus CMV (n=3), adenovirus (n=3), EBV (n=2), *M. tuberculosis* (n=1), Enterobacter sp. (n=1), gram negative (n=1), *P. aeruginosa* (n=1), rhinovirus (n=1), and *P. jirovecii* (n=1). Also, in the study by Cetinkaya et al., CMV (n=3) and parainfluenza 3(n=1) (4) were documented. Features of HLH were mostly associated with viral infections. However, the infections were most commonly bacterial in our cohort with 2 cases of disseminated BCGosis. Viral infection was identified in one patient only (17%) (P4). The increased risk of BCGosis in our setting is due to the lack of universal screening of SCID and effective universal vaccination with BCG vaccine to all newborns on Day 1 of life. However, the co-infections with viruses cannot be excluded due to lack of availability of molecular tests for viruses in our setting. Hence, in a setting of HLH with life threatening proven infections, either bacterial or viral, PIDs such as SCID need to be important differential diagnoses.

Most of the children with SCID have isolated lymphopenia at diagnosis and ALC in hemogram gives a clue to make a diagnosis. However, lymphopenia can also occur as a part of pancytopenia in HLH. In such cases, disproportionate reduction in T cell proportions, decrease in naïve T cells, altered CD4/CD8 ratio, and decreased lymphocyte proliferation provide laboratory clues toward underlying SCID (6). In our series, lymphopenia was seen in all patients; however, pancytopenia/bicytopenia (P1, P2, P6) was noted in 3 out of 6 cases. In these patients, an extremely low proportion of T cell percentage and decrease in naïve T cells provided vital clues toward underlying SCID.

Bode et al. (3) showed lower levels of serum soluble interleukin-2 receptor (sCD25) and higher ferritin levels in HLH associated with T-cell deficiencies compared to HLH in other PIDs. The authors also proposed that the ratio of ferritin: sCD25 ≥3 as a clue to suspect SCID/CID in a child with HLH. We could perform sCD25 levels in one patient and the levels were normal (P2). In this case, the ratio of ferritin and sCD25 was also high (19.3).

TABLE 5 | Review of literature of previously reported cases of HLH in SCID patients.

Author/Ref.	Genetic	Age (median age (IQR)/ Sex	Infections	HLH onset age	HLH features	Management of HLH	HSCTY/ N	Outcome
Chidambaram et al., 2020India (16)	Homozygous missense variation in exon 11 of the ADA gene	3 month/ F	CMV PCR positivity (bloodand urine)	3 months	Anemia, thrombocytopenia; high ferritin (13797 ng/dL); high serum triglycerides (532 mg/d)	IVIgDexamethasone; Ganciclovir	No	Expired
Bode et al., 2015 (3)	12 patients IL2RG:5 RAG 1:2 IL7RA:1 CD3E:1 Unidentified: 3	NA	CMV (n = 3), adenovirus (n = 3), EBV (2), M TB (n = 1), *Enterobacter* sp. (n = 1), gram-negative (n = 1), *P.* aeruginosa (n = 1) rhinovirus (n = 1), *Pneumocystis jirvoceii* (n = 1)	0.13-1.5 years	–	Corticosteroids alone or in combination with intravenous immunoglobulins, cyclosporine, or etoposide	NA	8 Expired 4 Survived
Cetinkaya, Turkey 2020 (4)	4 patients 2 RAG	2.5 months (2-5 months) M:F:3:1	CMV (n = 3) and parainfluenza 3 (n = 1)	4.5 months (2-22 months)	–	Corticosteroids alone or in combination with intravenous immunoglobulins, cyclosporine, or etoposide	Y (N = 1)	Expired 3 Alive:1 (HSCT)
Shi et al., 2020; China (17)	IL2RG gene (Exon 6: c.854G > A; p.Arg285Gln)	4 month/ M	*M. tuberculosis* *M. bovis*	4 months (Day 8 of HS)	Low fibrinogen (0.91 g/L); high ferritin 3235 ng/mL; high soluble CD25 cells (5182.51 pg/mL)	One intravenous etoposide (40 mg, IV in one dose) Dexamethasone (2 mg IV every 12 h)	No	Expired
Patirgolu et al., 2014 (14)	IL2RG gene; the novel mutation in exon 5 (c.595-1G>T)	3 month/ M	*Candida albicans* (blood culture) *Pseudomonas aeruginosa* (aspirated tracheal fluid)	3 month, (3rd week of HS)	Elevated transaminases, pancytopenia, high triglycerides and ferritin, low fibrinogen, hemophagocytic histiocytes in bone marrow	IVIG, broad-spectrum antibiotics, ATT	No	Expired
Grunebaum et al., 2000 (8)	IL2RG SCID	9 week/ M	NA	9 weeks (at onset)	Pancytopenia ; high triglycerides and ferritin, low fibrinogen; bone marrow of histiocytes with hemophagocytes	Etoposide and dexamethasone	No	Expired; Gram-negative septicemia
Alsalamah., 2015 (15)	Homozygous mutation in the CD3δ gene	6 month/ F	Adenovirus (nasopharyngeal swab); urine culture *Klebsiella pneumoniae*	6 months (at onset)	Anemia (Hb 88 g/L), thrombocytopenia; leukopenia; high ferritin (>100,000 μg/L), elevated triglyceride (12.24 mmol/L); hypofibrinogenemia (1.37 g/L) and high soluble IL-2 receptor (4683 U/mL)	Dexamethasone, IVIG, corticosteroids, and etoposide	No	Expired 2 weeks (ongoing HLH, refractory bleeding, and encephalopathy)
Suzuki et al., 2009 Japan (11)	NA	19 d/F	–		5/8 HLH	IVIG, corticosteroid, cyclosporine, etoposide, HSCT	Y	Expired
Schimid et al. (9)	(T–, B+, NK+) SCID Genetics NA	5 month/ M	Active EBV infection was diagnosed by quantitative PCR testing (675,000	6 days of HS	Anemia, thrombocytopeniaand leukocytosis; ferritin (5866 ng/ml) and triglycerides	Dexamethasone Cyclophosphamide Low dose	Y	Expired (ARDS Aspergillus in ET)

(Continued)

TABLE 5 | Continued

Author/Ref.	Genetic	Age (median age (IQR)/ Sex	Infections	HLH onset age	HLH features	Management of HLH	HSCTY/ N	Outcome
			genome equivalents/ 20,000 cells).		(241 mg/dl), erythrophagocytosis	Etoposide IVIG		
Dvorak et al., 2008 (10)	X-linked SCID	7 week/ M	Methicillin-sensitive *Staphylococcus aureus*At 7 weeks: Rhinovirus (nasopharyngeal swab); positive for *Enterobacter aerogenes* (blood culture)	7 weeks of life	Bone marrow active hemophagocytosis, elevated serum levels of ferritin (872 ng/ mL; normal <500 ng/mL), and soluble interleukin-2 receptor a chain (CD25) (9016 pg/mL), normal (239 to 7887 pg/mL),_ severe anemia, thrombocytopenia was moderate with a nadir of 36,109/L platelets	IVIG, 2-week course of cyclosporine (3 mg/kg/dose twice a day, adjusted for a goal level of 250 to 300 ng/mL): no response	Y	Well at 17 months
Klemann et al., 2017 (20)	ORAI1	6 weeks/ M	CMV infection was diagnosed based on of blood virus loads >100.000 IU/ml *Pneumocystis jirovecii* pneumonia	3 months of age at presentation	Pancytopenia; hyperferritinemia (5103ng/ml), hypertriglyceridemia (371 mg/dl = 4.1 mmol/l), hypofibrinogenemia (1.4 g/l) and elevated soluble CD25 (max. 4022 U/l).	Treatment with dexamethasone improved the HLH symptoms, but the patient relapsed upon tapering	Y	Expired (severe, CMV-associated pulmonary inflammatory complications)
Norris et al., 2009 (12)	IL2RG	5 month/ M	*Pneumocystis jirovecii.* Pneumonia. and parainfluenza virus type 3 The posttransplant course was complicated by numerous infections including persistent parainfluenza, *Corynebacterium* sp., *Enterococcus faecalis*, *Staphylococcus epidermis*, *Staphylococcus hominus*, *Staphylococcus haemolyticus*, *Serratia marcescens*, and *Clostridium difficile* colitis.	Post-HSCT	Pancytopenia; extensive lymphohistiocytic infiltrate with evidence of mild hemophagocytosis	4 weekly doses of rituximab (375 mg/m2) in addition to his immunosuppression with tacrolimus and prednisolone	Y	Chimerism continues to be 85% donor 20 months from second HSCT, and immunologic reconstitution is normal
Tucci, 2021 Italy (18)	ADA SCID	4 year/F	*Mycobacterium bovis Stenotrophomonas maltophilia* bacteremia, invasive pulmonary aspergillosis, adenovirus reactivation	Post 2nd HSCT (D +13)	Persistent fever, hepatosplenomegaly, high levels of triglycerides (383 mg/dL) and markedly elevated ferritin (18,000 mg/dL) and soluble IL2 receptor (16,809 pg/mL; BM morphology showed active hemophagocytosis	Methylprednisolone (2 mg/kg/day); high-dose immunoglobulins; Emapalumab max 6 mg/kg; surgical incision of the abscesses; anti-TB treatment	Y; 3 HSCT	Alive after 3rd HSCT Full donor chimerism at Day +100 post HSCT
Hashi et al., 2010 China (13)	Novel homozygous non-sense mutation of JAK3 (C623T; R175X)	5 month/ f	NA	Post HSCT (Day 18)	Cytopenia; BM aspiration revealed hypoplastic marrow with hemophagocytosis; high serum ferritin (715 ng/mL) and serum soluble IL-2 receptor level (3295 U/mL)	Etoposide 30 mg/m^2 and pulse methylprednisolone (30 mg/kg)	Y	Expired Day 32 post-HSCT (respiratory failure)

(Continued)

TABLE 5 | Continued

Author/Ref.	Genetic	Age (median age (IQR)/ Sex	Infections	HLH onset age	HLH features	Management of HLH	HSCTY/ N	Outcome
Singh et al., 2020 USA(19)	IL2RG	10 day/ M	Human herpes virus 6	10 days of life	Fever and pancytopenia with elevated ferritin (1251 ng/mL) and LDH (457 IU/mL) levels	Dexamethasone	Y	Alive

NA, Not available

Usually, it is the activated T lymphocytes that are involved in immunopathogenesis of HLH (21) (22). CD8+ T cell activation leads to interferon overproduction and macrophage activation. In patients with SCID and combined immunodeficiency, HLH-like manifestations occur despite severe T-cell deficiency/ impairment (3) (8). Lack of regulation of excess immune response by T cells due to defective IL-2/IL-2R system could possibly explain development of hyper-inflammatory complications in SCID (23).

Engrafted maternal T cells with oligoclonal expansion survive for a long duration in SCID (24). These activated maternal T cells can result in HLH (25) (26) (27). Dvorak et al. (10) showed that maternal CD8 T cell engraftment was a key driver for HLH. Similarly, HLH as a result of donor T-cell engraftment has also been shown to occur in children with SCID with post-HSCT (12) (13). Hence, host macrophage activation was presumably induced in response to donor/maternal engrafted T lymphocytes through immunoreaction to infections and/or alloantigens. The presence of these T cells is the likely source of the elevated circulating CD25 levels in such cases.

In simple terms, donor lymphocytes respond to host cells or resident infectious organisms, leading to IFN production and activation of host macrophages. In our series too, 3 children had GVHD/Omen like phenotype (P3,4,5). VNTR was performed in one child (P5) and maternal engraftment was ruled out.

Another pathogenesis for HLH is the activation of innate immunity. Gain-of-function mutations in NLRC4, a protein that activates an inflammasome, have been documented in cases of recurrent MAS (28–31). Further studies on innate immunity in cases of SCID with HLH-like manifestation can throw light into these new pathways.

Management of HLH-like manifestations in SCID involves identification of the infective trigger, aggressive management of infections, and early hematopoietic stem cell transplantation (HSCT). However, optimal immunomodulatory strategies for management of HLH in SCID is still not clear. Supportive therapy with IVIg and immunomodulatory therapies for HLH were used for management with hardly any success. Bode et al. (3) reported usage of IVIg in 4 patients, and steroid, etoposide, or HLH 1994/2004 (5, 32) protocol in 5 patients. Cetinkaya (4) reported 3/4 SCID patients (75%) died of HLH before HSCT and treatment with IVIg, dexamethasone, cyclosporine, and etoposide were tried. IVIg was used as a first line for managing of HLH in our series with no success. In our setting, none of the patients were able to reach the process of HSCT due to the serious illness and infections owing to delayed diagnosis. HLH can still occur post-HSCT, probably due to engraftment of donor cells and concurrent infections (12, 13) (18). Recently, emapalumab (18) has been successfully used in a child with recurrent HLH in SCID who underwent HSCT.

When short of HSCT, SCID is fatal. In the series by Bode et al. (3), 8/12 children with SCID HLH died, which is much higher than that of CGD (2/22). However, data on HSCT are not available. Cetinkaya (4) reported 3 of 4 SCID patients (75%) died of HLH before HSCT. In our study, all died due to delayed presentation and diagnosis, which probably must have led to fulminant uncontrolled infections and life threatening HLH. This again calls for the need of increasing awareness of SCID and its varied HLH-like presentation.

Various types of infections including viral, bacterial, and parasites have been shown to trigger HLH (33–36), However the exact mechanism of infections triggering HLH is unclear and the margin to differentiate HLH and infections causing sepsis is blurred. The probable mechanism of susceptibility to HLH could be uncontrolled infection with high antigen load resulting in cytokine storm and inhibiting apoptotic pathways. Also, a direct connection between the viral infection and inhibition of natural killer cell or T cell cytotoxicity was documented (35).

In children with SCID who developed HLH, there is also a possibility of the presence of concomitant genetic defects in any one of the genes associated with congenital HLH. However, NGS performed in our patients did not yield any variants in *PRF1* or *STX11* genes in 2 of them. Two patients who underwent a whole exome sequencing in a private laboratory did not reveal any pathogenic variants in the genes implicated for congenital HLH. Moreover, autopsy performed in 2 patients did reveal classical features of SCID such as thymic atrophy, lymphoid hypoplasia, and opportunistic infections apart from HLH. This suggests that the etiology of HLH-like manifestations in patients with SCID is likely acquired or secondary to infection. However, we have performed a whole exome in only 2 of our patients and, therefore, we cannot conclusively state that HLH in patients with SCID is only acquired and not congenital in origin.

5 CONCLUSION

HLH-like manifestations secondary to infections can be the presenting features of PID and diagnosis of SCID in such situations can be challenging. In such a setting, the presence of a suggestive family history, associated infections, and disproportionate T cell reduction in flow cytometry in such

settings provide clues to underlying SCID. Mortality is high in infants with SCID who had HLH-like manifestations and the role of immunomodulatory therapy in these cases is not clear. Establishment of genetic diagnosis can help in antenatal diagnosis in future pregnancies of the affected families.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

PV – Inception of idea, editing of the draft, clinical management, intellectual input, and final approval of the manuscript. GA – Preparation of the draft, clinical management, review of literature, final approval. RK – Preparation of the draft, laboratory work-up, final approval.

AS, SM, and JN – Clinical management, review of literature, final approval. AJ and DS – Clinical management, editing of the draft, final approval. MS, GK, and SSh– laboratory work-up, final approval. KG and SSr – laboratory work-up, editing of the draft, final approval. AR – editing of the draft, laboratory work-up, intellectual input, and final approval of the manuscript. SSi – editing of the draft, final approval. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.867753/full#supplementary-material

Supplementary Figure 1 | (A) Decreased surface expression of CD132 on lymphocytes in patient 1 (12.07%) as compared to control (40.53%). **(B)** Sanger plots of patient 1 and mother showing hemizygous state of mutation in the patient and heterozygous carrier state in the mother.

Supplementary Figure 2 | Family pedigree charts of patients with SCID and HLH-like manifestations (P1, P3, P5, P6).

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Case Report: Refractory Cytopenia With a Switch From a Transient Monosomy 7 to a Disease-Ameliorating del(20q) in a *NHEJ1*-Deficient Long-term Survivor

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We report the case of a male Pakistani patient with a pathogenic homozygous loss of function variant in the non-homologous end-joining factor 1 (*NHEJ1*) gene. The growth retarded and microcephalic boy with clinodactyly of both hands and hyperpigmentation of the skin suffered from recurrent respiratory infections. He was five and a half years old when he came to our attention with refractory cytopenia and monosomy 7. Hematopoietic stem cell transplantation was considered but not feasible because there was no suitable donor available. Monosomy 7 was not detected anymore in subsequent bone marrow biopsies that were repeated in yearly intervals. Instead, seven and a half years later, a novel clone with a del(20q) appeared and steadily increased thereafter. In parallel, the patient's blood count, which had remained stable for over 20 years without necessitating any specific therapeutic interventions, improved gradually and the erythropoiesis-associated dysplasia resolved.

Keywords: NHEJ1, refractory cytopenia, monosomy 7, del(20q), myelodysplastic syndrome, NHEJ1-deficiency

INTRODUCTION

Inherited bone marrow failure (IBMF) syndromes are genetically heterogeneous hematopoietic stem cell disorders that impede the adequate production of one or more blood cell lineages and consequently predispose affected individuals to the development of myelodysplastic syndromes (MDS) as well as myeloid malignancies (1–3). The responsible genetic defects often produce recognizable syndromes, whose main features are microcephaly, growth retardation as well as inconsistent other physical malformations and organ abnormalities. Such germline alterations comprise pathogenic variants in genes that encode for transcription factors such as GATA2, RUNX1 and ETV6, products that are involved in telomere maintenance (dyskeratosis congenita),

ribosomal biogenesis (Blackfan-Diamond anemia) and maturation (Shwachman-Diamond syndrome; SDS), DNA maintenance and repair (Fanconi anemia), protein folding and trafficking (severe congenital neutropenia) as well as in the regulation of cell proliferation and apoptosis (SAMD9/9L) (3–9). In many instances characteristic clinical, laboratory and hematologic parameters alone will already suffice to identify the respective syndrome. Nevertheless, the large number of not only genes but also of possible types of pathogenic variants that need to be considered even in such well-defined syndromes requires a thorough molecular genetic clarification to obtain a precise diagnosis. In case of overlapping symptoms and/or if no pathogenic sequence abnormalities are found, an even broader screening approach with whole exome sequencing may be necessary to identify rarer or even previously not considered causes of such diseases.

Herein we report the extraordinary disease development in a patient with distinctive yet originally difficult to interpret phenotypic features, in whom, after a long and challenging diagnostic odyssey, we finally succeeded to secure a homozygous pathogenic variant in the *NHEJ1* gene as the responsible germ line defect.

CASE REPORT

The now 26-year-old patient [case #17; Table 2 in our previous publication (10)] is the son of consanguineous Pakistani parents, who were healthy and had normal blood counts. One sister was healthy, a second sister suffered from hepatitis C, but had normal blood counts. He had been prone to infections, primarily recurrent bronchitis, since birth and was first seen in our clinic when he was five and a half years old with fever and coughing. He was growth retarded and had a microcephaly (<3rd percentile), clinodactyly of both hands and hyperpigmented skin above the knees and elbows. Apart from his microcephaly, imaging of the head, the thorax and the abdomen remained inconspicuous. Screening for causative infectious agents, including *Mycobacterium tuberculosis*, was unrewarding. Immunological analyses that were performed during the course of the disease revealed IgA deficiency and B-lymphocytopenia (Details are provided in **Supplementary Table 1**). Hematologic analysis at first presentation revealed a white blood cell count of 1.47 x10^9/L with an absolute neutrophil count of 0.76 x10^9/L and an absolute lymphocyte count of 0.47 x10^9/L, a hemoglobin level of 9.2 g/dL, MCV of 75fl, and a platelet count of 109 x10^9/L. Data on the long-term course of hematological parameters are provided in **Supplementary Figure 1**. A bone marrow (BM) examination disclosed reduced cellularity of all three lineages with an erythropoiesis-restricted dysplasia that, together with the presence of a fluorescence *in situ* hybridization (FISH)-verified monosomy 7 in 18% of the analyzed nuclei, was consistent with the diagnosis of a hypocellular myelodysplastic syndrome in form of a refractory cytopenia (11, 12). Although the appearance of monosomy 7 in IBMF is strongly indicative of an underlying SAMD9/9L or GATA2 deficiency, these disease-promoting germline causes were not known at that time (6, 13, 14).

We discounted the most likely causes of the patient's problems, namely Fanconi anemia, Nijmegen breakage syndrome and dyskeratosis congenita with an originally negative diepoxybutane (DEB) breakage analysis in the one, molecular testing in the other and based on clinical parameters in the latter, respectively. Another DEB test that was performed at the age of 14 years showed an elevated chromosome breakage (4.78, normal <0.6) but without the pathognomonic Fanconi anemia-specific chromatid exchange figures. Moreover, a cell cycle analysis of the patient's skin fibroblasts, which was kindly performed in the Department of Human Genetics, University of Würzburg, Würzburg, Germany, lacked the otherwise typical G2 cell cycle blockage (15). Normal pancreatic laboratory parameters also excluded a less likely Shwachman-Diamond syndrome and lymphocyte immunophenotyping the presence of paroxysmal nocturnal hemoglobinuria clones that are seen in up to 38% of refractory cytopenia cases (16, 17). Since the appearance of monosomy 7 in patients with such syndromic features and pancytopenia often precedes and forecasts the transformation into myeloid malignancy, the current EWOG-MDS 2006 protocol (NCT00662090) recommends as the treatment of choice to perform a hematopoietic stem cell transplantation (HSCT), which was not feasible in this case because we did not find a suitable donor. However, contrary to all odds, his blood counts remained stable at this low level over the following years, and he did not even require any transfusions. He had only a mild obstructive ventilation disorder and continued to suffer from recurrent but well-manageable pulmonary infections. Although we did not find the clone with the monosomy 7 anymore in the follow-up BM examination three months later, another clone with a deletion of the long arm of chromosome 20, del(20q), emerged seven and a half years later, when the patient was 13 years old. The respective FISH analyses were performed with a del(20q)-specific dual-color probe set (leicabiosystems.com). Subsequent BM examinations that were executed in yearly intervals thereafter showed that this abnormal clone steadily increased from originally 15% to 90% within the following six years and then dropped again to 68% a year later, at which point the erythropoiesis-restricted dysplasia also had resolved. Nonetheless, even two years later, we found that 34% of the peripheral blood cells still descended from this del(20q) clone, even though the patient was in good clinical condition and with a notably improved white blood cell count of 3.06 x10^9/L, an absolute neutrophil count of 1.27 x10^9/L, an absolute lymphocyte count of 1.53 x10^9/L, a hemoglobin level of 11.6 g/dL and a platelet count of 82 x10^9/L. The hematological parameters, however, display a fluctuating pattern during course (**Supplementary Figure 1**).

GENETIC ANALYSES

When our patient was 21 years old, we finally succeeded to identify the genetic cause of his physical and hematological

problems with our next-generation targeted sequencing hematology panel. This approach uncovered a unique and hitherto undescribed pathogenic homozygous missense variant (NM_024782.2:c.236T>C, p.Leu79Pro; GRCh37) in exon 3 of the *NHEJ1* ("non-homologous end-joining factor 1", also known as *XLF* "*XRCC4*-like factor" or Cernunnos, OMIM *611290) gene on chromosome 2(q35) (10). The pathogenic variant had a CADD v.1.3 ("combined annotation dependent depletion") score of 31 and was thus classified as being intolerable (**Figure 1**, left) (19). Segregation analysis confirmed the heterozygous carrier status of both parents (**Figure 1**, right).

At this time, immunophenotyping of peripheral blood cells revealed a selective CD19+ B-cell lymphopenia with a nadir of 0.02 x10^9/L. Immunoglobulin G and M levels (IgA deficiency) as well as the results of the stimulated T cell proliferation tests were normal, although the vaccine-dependent reactivity was moderately reduced. Since an ongoing telomere length shortening of hematopoietic stem cells had been previously reported to contribute to the development of cytopenia in *NHEJ1*-deficient cases, we had this parameter examined in the Department of Pediatric Hematology and Oncology, University of Freiburg, Germany (20, 21). Comparison of his telomere repeat copy number with that of reference samples confirmed that the telomeres in his peripheral blood cells were, with a ratio of 0.57, indeed severely shortened, namely to an extent that was below the first percentile (0.61) of a healthy control cohort (n=90).

To determine the exact location and extension of the hematopoiesis-restricted del(20q), we performed a CytoScan™ HD array analysis. This array comprises 2,670,000 markers, including 750,000 single nucleotide polymorphism probes (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA). We obtained the data from a commercial service provider and analyzed them in-house with the Chromosome Analysis Suite (ChAS; Applied Biosystems™, ThermoFisher Scientific) software package version 4.1 as described previously in detail (22). Overall, six percent of the autosomal genome was homozygous, indicating consanguinity in the family. In addition to the del(20q) we noted seven such consanguinity-associated homozygous regions that were larger than 3Mb, namely on chromosomes 2(q34-q37.2), which contained the *NHEJ1* pathogenic variants, 3(p12.3-q13.31), 7(q34-q36.1), 8(q22.1-q22.3), 9(p21.3-q21.32), 14(q11.2-q12) and 21(q11.2-q21.3) as well as a unique 78kb duplication at chromosome 20(p11.2) that partially disrupted the *GINS1* gene (**Supplementary Table 2**). Biallelic loss of function pathogenic variants in this gene cause another very rare, phenotypically very similar immunodeficiency syndrome (IMD55; OMIM #617827) with intrauterine growth retardation, chronic neutropenia, and natural killer cell deficiency (23). The 22,124 Mb large interstitial del(20q) encompassed nearly the entire long arm and removed 244 genes that are contained in this region (**Figure 2**). Of the three genes that are of specific relevance in the context of an acquired del(20q), two, namely the imprinted *L3MBTL1* and *SGK2* genes were deleted, whereas the *EIF6* gene was not (**Figure 2**) (24–28).

DISCUSSION

The case presented herein is only the second NHEJ1-deficient patient who reached adulthood without transplantation (29). Despite the early onset of a refractory cytopenia with a transient monosomy 7 and the emergence and subsequent expansion of a del(20q) clone many years later, his disease neither progressed into a *bona fide* myeloid malignancy nor did it require any specific therapeutic care during the now overall 20 year-long

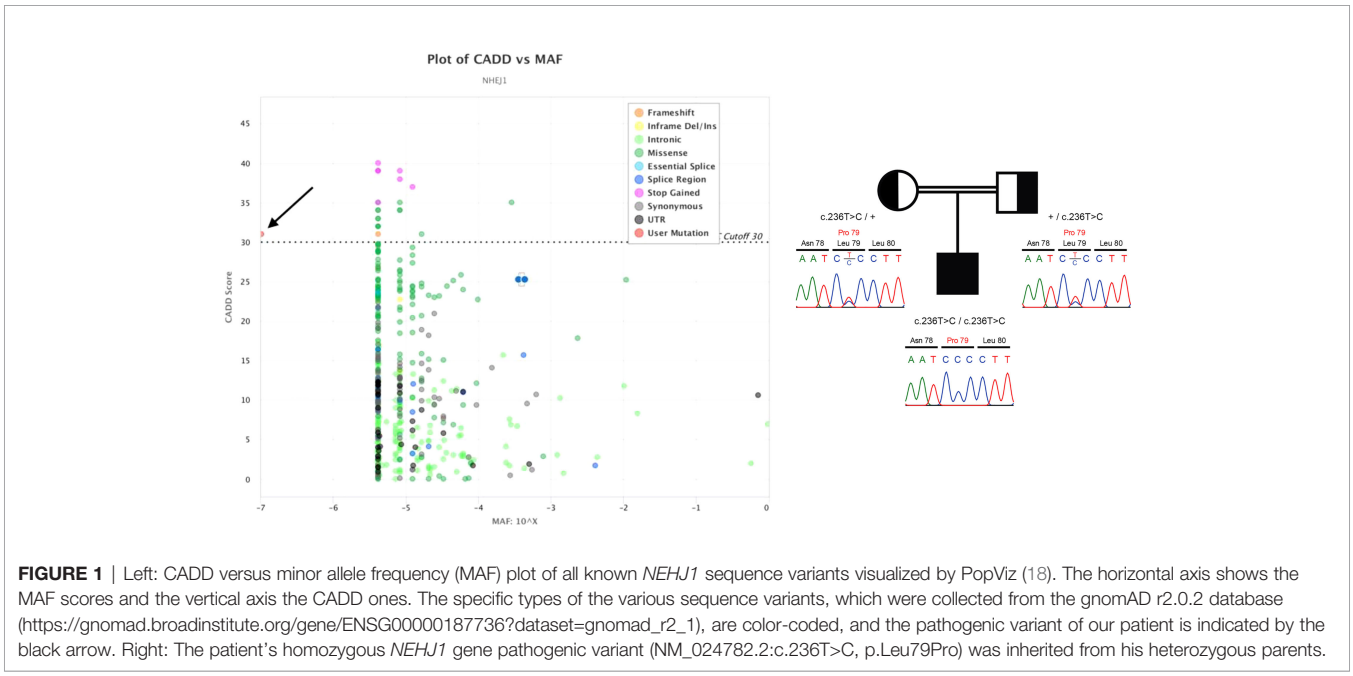

FIGURE 1 | Left: CADD versus minor allele frequency (MAF) plot of all known *NEHJ1* sequence variants visualized by PopViz (18). The horizontal axis shows the MAF scores and the vertical axis the CADD ones. The specific types of the various sequence variants, which were collected from the gnomAD r2.0.2 database (https://gnomad.broadinstitute.org/gene/ENSG00000187736?dataset=gnomad_r2_1), are color-coded, and the pathogenic variant of our patient is indicated by the black arrow. Right: The patient's homozygous *NEHJ1* gene pathogenic variant (NM_024782.2:c.236T>C, p.Leu79Pro) was inherited from his heterozygous parents.

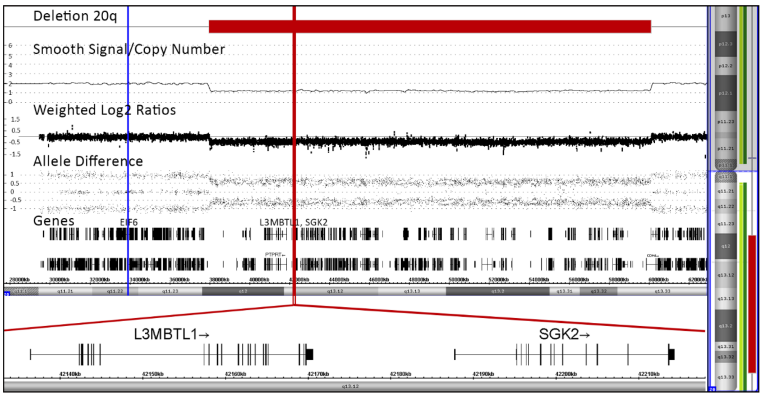

FIGURE 2 | Array pattern of long arm of chromosome 20 showing the interstitial deletion with the following bordering coordinates: chr20:37948298-60071887 (hg19). The deletion extends over 22,124 Mb and encompasses 244 genes, a list of which is provided in the **Supplementary Table 2**. The location of the three potentially most relevant genes, *EIF6, L3MBTL1* and *SGK2*, are indicated with blue and red lines, respectively (24–28). The orientation and detailed structure of the two imprinted ones in the deletion are shown in the blown-up section on the bottom part of the Figure.

observation period. Given that even his genetic condition and his hematologic disease alone would have sufficed to transplant him, the lack of a suitable donor was in retrospect a stroke of luck for both the patient as well as the treating physicians (11, 14, 30, 31).

The *NHEJ1* gene encodes one of the components of the principle nonhomologous end-joining repair pathway, whose other constituents are the products of *LIG4* (encoding DNA ligase IV), *PRKDC* (encoding DNA-PKcs), *DCLRE1C* (encoding Artemis), and *XRCC4* (encoding XRCC4) (32–35). This system is not only responsible for the repair of double strand breaks but also for the appropriate execution of V(D)J recombination (32–35). Thus, pathogenic variants in any of these genes increase the radiosensitivity of affected tissues and disturb V(D)J as well as class switch recombination processes. The ensuing problems produce developmental defects in form of a growth delay, microcephaly and dysmorphic facial features as well as various types of (severe) combined immunodeficiencies with differing degrees of B and T cell lymphocytopenia (9, 29, 32–37). In addition, such germline pathogenic variants also predispose affected individuals to the development of autoimmune diseases, lymphomas, bone marrow failure as well as lymphoid and occasionally also myeloid leukemias (9, 34, 38, 39). Although we did not perform any functional assays or radiosensitivity studies, the elevated chromosome breakage in the second DEB test at least provides some evidence that the double strand breakage repair was indeed impaired.

Since the overlapping actions of the *PAXX* and *ATM* gene products can to some extent compensate functional impairments of the NHEJ1 protein, one would not expect that it plays such a vital role as, for instance, that of the *LIG4*-encoded DNA ligase IV (34, 35, 40, 41). Nevertheless, NHEJ1 deficiencies still affect the respective repair and recombination processes quite profoundly, so that the ensuing clinical consequences usually resemble those of the otherwise more severe *LIG4* defects (34). Less than 50 cases with bi-allelic *NHEJ1* loss-of-function pathogenic variants have so far been documented in the literature (21, 29, 31, 32, 36, 37, 42–44). The heterogeneous phenotypes and variable clinical courses of patients with different but also identical pathogenic variants severely impede any attempts to establish an even only approximate genotype-phenotype relationship, not least also because the effects of the diverse pathogenic variants are also cell type-specific and differentiation stage-dependent (29, 42, 45). Cases in point are, for instance, the progressive lymphocytopenia as well as bone marrow aplasia in some NHEJ1-deficient individuals, which almost certainly can be put down to a premature aging of hematopoietic stem cells (21, 45, 46). This problem is most likely triggered by the inability of the affected stem cells to properly repair continuously accumulating double strand breaks as well as by a pathogenic variant-triggered decrease in telomerase activity that, as also seen in our patient, leads to a gradual loss of telomeres (21, 45, 46). We are aware of altogether five patients with such a bone marrow aplasia, all of whom were transplanted and, all but one, were alive at the time of reporting (21, 31, 32).

Abnormalities of chromosome 7 are the most common acquired genetic changes in childhood myelodysplastic syndromes. They comprise the loss of an entire copy as well as various structural abnormalities in form of deletions, translocations and isochromosomes of its long arm (14, 47). A monosomy 7 is seen in virtually all types of IBMF, but the frequency of its occurrence varies depending on the underlying primary germ line defect (14, 47). The two most common ones are the SAMD9/SAMD9L and GATA2 syndromes (6, 7, 14). Together they account for at least 50% of pediatric MDS with monosomy 7, although the disease emerges primarily in younger children in the former and primarily in adolescent ones in the latter (6, 7, 14). Moreover, monosomy 7 is also the most common alteration in patients with a hypocellular refractory cytopenia, although it is seen in only approximately nine percent of them (11, 12, 30). The only other case that is vaguely comparable to ours is one with a *LIG4* germline pathogenic variant and an MDS-related deletion of 7q (48).

The emergence of a monosomy 7 in patients with a hypocellular refractory cytopenia usually concurs with a high probability of disease progression. Nevertheless, in some of the patients the abnormal clone may disappear again and thereby lead to a spontaneous improvement or even disease remission. In the meantime, such transient forms of monosomy 7 are well documented in the literature (13, 49–54). In case of SAMD9/9L-associated disorders, monosomy 7 always results from the nonrandom loss of the homologue that carries the respective *SAMD9/9L* germline defect (6, 7, 13, 14, 47, 55, 56). These clones may occasionally experience a spontaneous duplication of the remaining homologue that carries the wild-type *SAMD9/9L*, which will then promote the functional normalization of the bone marrow (6, 7, 13, 14). Since the *NHEJ1* gene is located on chromosome 2 rather than on chromosome 7, we neither expected nor detected such a repair process-associated uniparental disomy 7 in the array analyses and conclude that the monosomic clone had no competitive advantage and simply got lost again (6, 7, 14, 51, 52).

The subsequent appearance and gradual increase of another clone with a del(20q), seven and a half years later, concurred with an improvement of his blood counts as well as with the continuous resolution of the erythropoietic dysplasia. A del (20q), either alone or in combination with other chromosome abnormalities, is seen in many different types of myeloid malignancies of all age groups (57, 58). Incidental observations in non-myeloid malignancies and unexplained cytopenia, however, prove that it is not always a *bona fide* indicator of malignancy and that in such instances the progression to MDS is extremely low (57–59). In children, a solitary del(20q) occurs in an age-dependent manner almost exclusively in those who suffer from a SDS (27, 28, 60–63). With a prevalence of 20% it is also the most common acquired abnormality followed by an isochromosome 7(q10), which is seen in 10% (63). Both these changes may occur either alone, simultaneously, sequentially, or even only transiently, but irrespective of the specific constellation, cases affected by either abnormality hardly ever progress into a genuine myeloid malignancy (28, 63, 64). The cytopenia of SDS patients with a del(20q) remains as stable and the dysplastic alterations as mild as the ones that we observed in our NEJH1-deficient case. The positive influence of a del(20q) on disease development has been put down to the facts that affected totipotent stem cells not only maintain their multipotential differentiation capacity but that, in addition, they also gain a selective advantage (60). However, the impact of such abnormal stem cells in individual settings is virtually impossible to predict, because their destiny is primarily governed by their competitive fitness, which in turn is to a large extent also influenced by a variety of individual host factors and, not least, of course by the type of the preexistent germline defect.

The three genes that are currently in the focus of interest in this context are *L3MBTL1* and *SGK2*, which were lost, and *EIF6*, which was retained in our case (24, 26, 65). *L3MBTL1* and *SGK2* are two paternally expressed imprinted genes that encode a transcriptional repressor and a serine/threonine protein kinase, respectively. These genes are in the minimal commonly deleted region of 30 adult cases with a solitary del(20q), but also lost in SDS patients (26, 28, 57, 66). *In vitro* experiments revealed that their regulatory interactions vary in different hematopoietic lineages and successive stages of differentiation (25, 67, 68). Their coordinated silencing maintains megakaryopoiesis and enhances erythropoiesis but does apparently not equip affected stem and early progenitor cells with any selective advantage (25, 69). Attempts to associate the parental origin of the deletion with their anticipated allele-specific expression patterns produced no clear results and therefore also no coherent picture. Explanations for the various confusing discrepancies ranged from problems that may have arisen from inadequate clone sizes, from admixture of normal cells, from loss of imprinting and, most intriguing, also from the concurrence of two distinct clones, in which one lost the maternal and the other one the paternal allele (25, 26, 67, 69–71). The best clinical, albeit indirect evidence that a clonal del(20q) indeed enhances erythropoiesis derives from polycythemia cases (69). Another notable example relates to the observation that the hemoglobin concentrations and red blood cell counts of SDS patients with a del(20)(q) are higher than those without such a deletion (26).

EIF6, the retained gene, encodes the eukaryotic translation initiation factor 6, which is an essential ribosome chaperone protein (65, 72). To allow the formation of the mature 80S ribosome, this factor must first be released from the pre-60S ribosome subunit by the SBDS protein. Thus, *EIF6* inactivating point mutations or deletions become only relevant in patients with preexisting *SBDS* mutations, in whom they will help to reestablish a normal SBDS : EIF6 protein ratio, which improves the maturation and translational capacity of the ribosomes and consequently also enhance the competitive fitness of the affected hematopoietic cells (65, 72). Deletions of the *EIF6* locus in other forms of myeloid malignancies might therefore be functionally irrelevant and merely coincidental.

Taken together, the indolent development of a chromosomally abnormal refractory cytopenia in our patient with an already preexistent severe DNA repair defect is quite remarkable but not a unique phenomenon. Such observations reinforce the growing awareness that the emergence of abnormal clones in IBMF syndromes is not deterministic of malignant transformation. Instead, as we show herein, it can also stabilize and improve the disease process in a quite unexpected manner. The big challenge that derives from this insight is now the need to delineate harmless or even favorable abnormalities from undisputable malignant ones. The definition of even only approximate distinguishing criteria will significantly help to advance clinical decision processes, especially whether and for how long one can rely on a "watch and wait" strategy or whether at all and when one should pursue a more aggressive treatment, such as stem cell transplantation (65, 73–75).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization and supervision: LK, KB, OAH; manuscript drafting and writing: FP, OAH; methodology: RJH, PZ, KN; data analysis and interpretation, RJH, PZ, KN, OAH, KB; provision of clinical information and patient care: FP, WN, MND, LK; illustrations: RJH, PZ, KN. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.869047/full#supplementary-material

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