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IMMUNE DEFICIENCY AND DYSREGULATION (DP HUSTON AND C KUO, SECTION EDITORS)

Diagnostic Tools for Inborn Errors of Human Immunity (Primary Immunodeficiencies and Immune Dysregulatory Diseases)

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Abstract

Purpose of Review The purpose of this review is to provide an overview of diagnostic testing in primary immunodeficiency and immune dysregulatory disorders (PIDDs), particularly focusing on flow cytometry and genetic techniques, utilizing specific examples of PIDDs.

Recent Findings Flow cytometry remains a vital tool in the diagnosis and monitoring of immunological diseases. Its utility ranges from cellular analysis and specific protein quantitation to functional assays and signaling pathway analysis. Mass cytometry combines flow cytometry and mass spectrometry to dramatically increase the throughput of multivariate single-cell analysis. Next-generation sequencing in combination with other molecular techniques and processing algorithms has become more widely available and identified the diverse and heterogeneous genetic underpinnings of these disorders.

Summary As the spectrum of disease is further clarified by increasing immunological, genetic, and epigenetic knowledge, the careful application of these diagnostic tools and bioinformatics will assist not only in our understanding of these complex disorders, but also enable the implementation of personalized therapeutic approaches for disease management.

Keywords Flow cytometry · Genomics · Primary immunodeficiencies · Immune dysregulatory diseases · Mass cytometry · Diagnostic immunology

Introduction

Inborn errors of human immunity, or primary immunodeficiencies and immune dysregulatory disorders (PIDDs), comprise a broad group of genetic disorders with wide phenotypic variability. Non-genetic phenocopies of immunodeficiencies, as well as other types of immune dysregulatory diseases, could potentially be included in such a discussion of PIDDs, but constraints of space preclude discussing these in this review. The diagnosis of PIDDs requires a relevant clinical phenotype, physical exam, and careful family history (Fig. 1a), and other tools, such as newborn screening, can facilitate early diagnosis in asymptomatic individuals. An efficient, stream-lined diagnostic work-up is important for a timely diagnosis, but remains challenging due to heterogeneity in disease presentation, even among patients with the same genetic pathogenic variant. As knowledge of human immunology continues to improve, diagnostic algorithms will evolve and become more robust.

Here we present a review of current diagnostic modalities as they relate to PIDDs (Table 1). Flow cytometry, mass cytometry, newborn screening techniques, and genetic testing are discussed in the context of specific disorders and broader PIDD screening. Additionally, given the large datasets generated by genome sequencing, a brief discussion of data processing and algorithms is also reviewed.

This article is part of the Topical Collection on *Immune Deficiency and Dysregulation*

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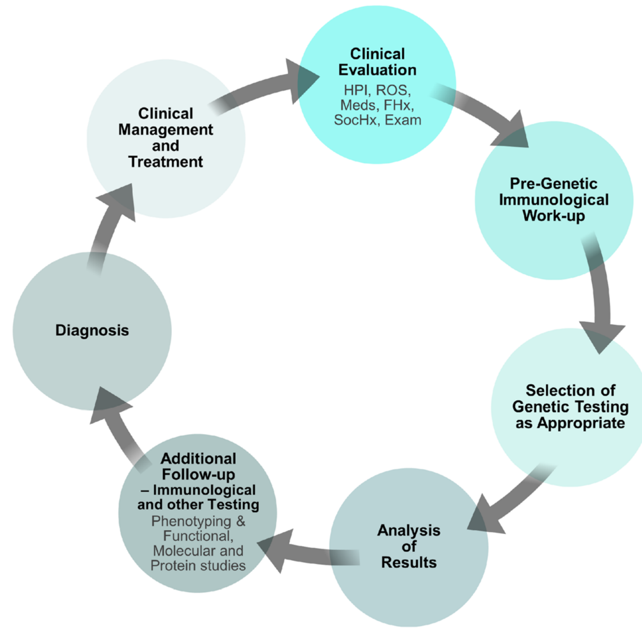

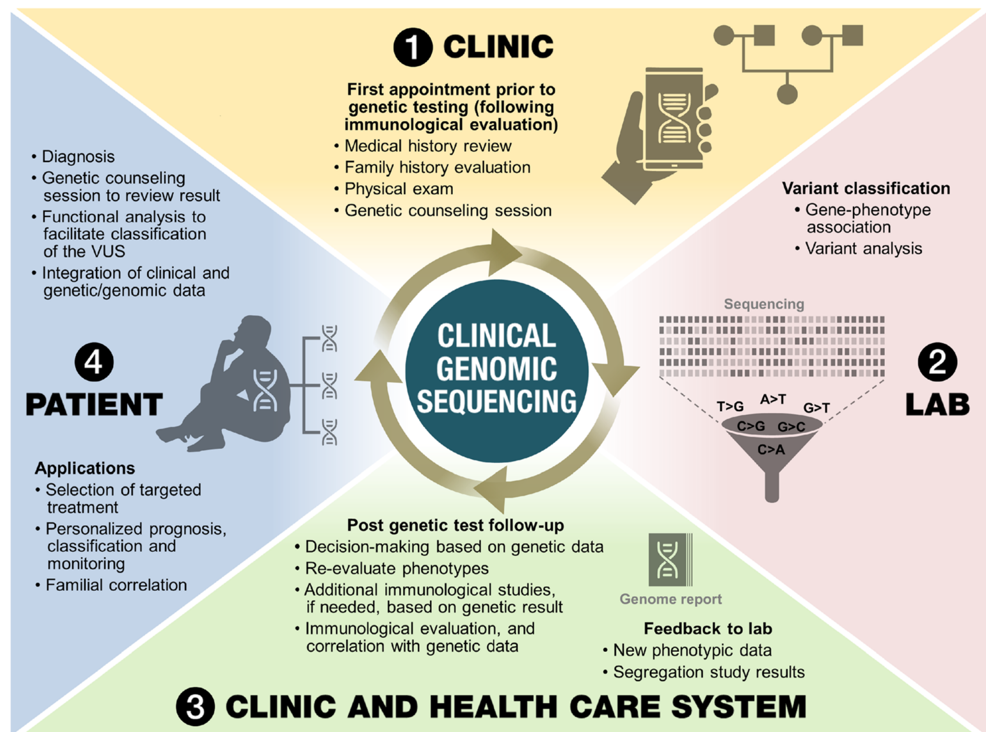

Fig. 1 **a** Schematic for diagnostic evaluation of primary immunodeficiencies. The various components that should be included in the diagnostic work-up of a patient with a suspected primary immunodeficiency are provided in this chart. **b** A rational approach to selecting and interpreting genomic analysis for primary immunodeficiencies. This facilitates integration of clinical data with immunological and genetic analyses to establish a diagnosis

Flow Cytometry

Application of Flow Cytometry in Diagnostic Testing in PIDD

Flow cytometry is one of the mainstays of clinical immunological investigation [1, 2]. Its utility ranges from immunophenotyping, disease-specific protein expression, functional assays, measurement of telomere length, binding partner assays, and evaluation of immune reconstitution, and immune competence. An overview of several PIDDs is provided to highlight the range and depth of flow cytometry assays employed in the diagnosis of PIDDs.

Table 1 Tabulation of diagnostic tools, including specific test(s), clinical/diagnostic utility, and availability

Assays	Utility	Availability of testing (clinical (C), reference (R), research (RS))
CBC with differential	Basic immune work-up for possible PIDD	C
T, B, and NK cell lymphocyte subset quantitation	Basic immune work-up for possible PIDD	C, R
Quantitative immunoglobulins (IgG, IgA, and IgM, with IgE and IgD based on clinical context)	Basic immune work-up for possible PIDD	C
Non-disease-specific assays		
B cell subset phenotyping	CVID; humoral PIDD; combined immunodeficiency	C, R
T cell subset phenotyping	Newborn screen follow-up; SCID; DiGeorge syndrome; T cell lymphopenia; cellular/combined PIDD	C, R
NK cell subset phenotyping	NK cell primary immunodeficiency, GATA-binding protein 2 (GATA2) deficiency, XMEN among others	C, R
Dendritic cell phenotyping	Dendritic cell deficiencies	R
Lymphocyte/T cell proliferation	Global or antigen-specific evaluation of T cell function	R
Regulatory T cell (Treg) quantification	Immune dysregulation with autoimmunity conditions; CHAI; LATAIE among others	R
Treg function	Monogenic inflammatory bowel disease, other immune dysregulatory diseases	R, RS
TLR function	Innate immune deficiencies	R
TCRVβ analysis by flow cytometry	T cell repertoire diversity	R
Disease-specific testing		
ALPS screen	Autoimmune lymphoproliferative syndrome	R
CD40L flow	X-linked hyper-IgM syndrome	R
BTK flow	X-linked agammaglobulinemia	R
DHR flow	Chronic Granulomatous Disease	C, R
LAD1 flow	Lymphocyte adhesion deficiency, type 1	R
DOCK8 flow	Detector of cytokinesis 8 (DOCK8) deficiency	R
WAS flow	Wiskott-Aldrich syndrome	R
SAP flow	X-linked lymphoproliferative syndrome, type 1	R
XIAP flow	X-linked lymphoproliferative syndrome, type 2 (XIAP deficiency)	R
CTLA-4/LRBA flow	CHAI; LATAIE	R
DNA repair radiosensitivity by flow	Ataxia-telangiectasia; radiosensitive-SCID; other disorders of DNA repair	R, RS
Mass cytometry	In-depth phenotypic and functional characterization of the immune system	RS
Molecular testing		
TREC	Newborn screening for SCID; monitoring of immune reconstitution post-hematopoietic cell transplant; diagnosis of other cellular and/or combined PIDD	R
TCRVβ spectratyping	T cell receptor repertoire diversity in SCID; other cellular combined PIDDs; immune reconstitution after hematopoietic cell transplant	R
Single gene/known variant analysis		R
Targeted gene sequencing (TGS)	In the setting of a defined clinical phenotype	R
Whole exome sequencing (WES)	Consider if negative TGS or broad clinical phenotype	R, RS
Whole genome sequencing (WGS)	Consider if negative TGS and WES	R, RS
Transcriptome analysis	Follow-up on prior DNA analysis	RS

PIDD primary immunodeficiency and immune dysregulation disorders; *CVID* common variable immunodeficiency; *XMEN* X-linked immunodeficiency with magnesium defect, Epstein-Barr virus infection, and neoplasia; *CHAI CTLA-4* haploinsufficiency with autoimmune infiltration; *LATAIE* LRBA deficiency with autoantibodies, regulatory T cell defects, autoimmune infiltration, and enteropathy; *XIAP* X-linked inhibitor of apoptosis; *SCID* severe combined immunodeficiency; *TREC* T cell receptor excision circle

Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome (ALPS) is a PIDD characterized by defective lymphocyte apoptosis leading to chronic noninfectious, non-malignant lymphoproliferation, elevated "double-negative" T (DNT) cells ($CD3^+TCR\alpha\beta^+CD4^-CD8^-$), hypergammaglobulinemia, autoimmune cytopenia(s), and an increased risk for lymphoma [3]. The most common form of ALPS is due to a heterozygous germline pathogenic variant in the *FAS* gene [3], but homozygous pathogenic variants in *FAS* as well as variants in FAS ligand (*FASL*) and Caspase 10 (*CASP10*) have also been identified [4]. Approximately 20% of patients remain without a genetic diagnosis [4], though this number may include ALPS-like conditions, which can have a similar clinical phenotypes [5]. Revised diagnostic criteria for ALPS, as established by the 2009 NIH International Workshop, include the presence of two required criteria (greater than 6 months of lymphadenopathy in the absence of infection or malignancy, and elevated DNT cells in the absence of lymphopenia), as well as at least one primary accessory criterion (defective lymphocyte apoptosis or a known pathogenic variant in *FAS*, *FASL*, or *CASP10*). A probable diagnosis can be rendered in the presence of both required criteria and at least one secondary accessory criterion (supportive family history, autoimmune findings with polyclonal hypergammaglobulinemia, typical pathological findings on tissue immunohistochemical analysis, and/or elevated associated markers in the plasma and/or serum) [3].

To increase the positive yield of genetic testing (and for identification of patients lacking in *FAS* gene pathogenic variants), the use of additional flow cytometry-based markers may be of clinical use. Immunophenotyping has aided in the immunological characterization of ALPS, revealing lymphocyte subset alterations. Changes noted have included increased γδ-DNT cells, total B cells, and $CD5^+$ B cells, and reduced $CD3^+CD25^+$ T cells in patients with pathogenic variant-confirmed, symptomatic ALPS versus asymptomatic and normal controls [6]. Additionally, significantly higher levels of $CD3^+$/HLA^-DR^+ T cells and decreased levels of $CD3^+CD25^+$ T cells have helped distinguish symptomatic from asymptomatic patients with a common genetic variant [6]. As these two findings occur independently, a ratio of $CD3^+CD25^+$ to $CD3^+$/HLA^-DR^+ T cells of 1 or less offers a 83% sensitivity and 90% specificity to distinguish clinical ALPS [6]. Bleesing et al. have reported on the use of B220 on DNT cells in ALPS cases [7–9]. B220 is an unusual isoform of O-glycosylated CD45 present on a small subset of memory B cells in humans, which may be indicative of incomplete targeting for apoptosis [8]. ALPS patients with either *FAS* or *FASL* pathogenic variants also express B220, which can be detected by flow cytometry [9].

Soluble markers, such as vitamin B12 and soluble FAS ligand, have also been shown to be predictors of *FAS* pathogenic variants in patients presenting with clinical features of ALPS [10••]. Rensing-Ehl et al. examined 98 patients with suspected ALPS for *FAS* pathogenic variants as well as several markers, including DNT, vitamin B12, and soluble FAS-ligand [10••]. In this cohort of patients, vitamin B12 and soluble FAS ligand were better predictors of *FAS* pathogenic variant than traditional DNT cells, particularly when used together, in combination [10••]. The authors developed a web-based probability calculator to help determine the clinical need for genetic *FAS* variant analysis [10••].

While expansion of the DNT cells characteristically is comprised of αβ-T cell receptors, a new report raises the possibility of involvement of γδ-DNT cells [11]. Two unrelated patients with treatment-refractory ALPS symptoms harboring different heterozygous pathogenic variants in *FAS* demonstrated significant expansion of γδ-DNT lymphocytes, as opposed to αβ-DNT cells, marking the first known report of an alternative TCR expansion resulting in ALPS-FAS [11]. The implications of this finding have yet to be determined. Other recently reported findings in ALPS diagnostics include a novel heterozygous frameshift variant in *TNF-α-induced protein 3* (*TNFAIP3*) encoding the protein A20 [4]. Polymorphisms in *TNFAIP3* are known to be associated with autoimmune conditions, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, and Crohn disease, while loss-of-function pathogenic variants result in haploinsufficiency of A20 and associated early-onset autoinflammatory disease [12]. *TNFAIP3* polymorphisms have also been associated with different types of lymphoma (Hodgkin, diffuse large B cell lymphoma, and mucosa-associated lymphoma) [4]. Finally, an index case of acute pancreatitis in a patient with genetically confirmed ALPS led to the diagnosis of concurrent IgG4-related disease. van de Ven et al. screened an additional group of 18 patients with ALPS-FAS and found that 22% demonstrated elevated IgG4, but were clinically asymptomatic with no end-organ involvement [11].

DOCK8 Deficiency

Dedicator of cytokinesis 8 (DOCK8) deficiency is an autosomal recessive (AR) cause of hyper-IgE syndrome, characterized by recurrent infections, chronic dermatitis, and food allergy with laboratory evidence of eosinophilia and elevated IgE. Initially, the clinical features may overlap with atopic dermatitis causing diagnostic challenges. Diagnosis may be confirmed by immunoblotting for DOCK8 protein; however, this test is not widely available in clinical laboratories. Therefore, intracellular flow cytometry can be a rapid and convenient modality for the assessment of DOCK8 deficiency. This assay also enables post-transplant monitoring and evaluation of carrier status in relatives, and has a much faster,

more economical turnaround time than genetic evaluation [13•]. While the technique is sensitive, missense variants that allow for nonfunctional or near normal DOCK8 protein expression will not be detected by this method [13•].

Beyond assessment of DOCK8 protein, assessment of lymphocyte subsets may be of use. Janssen et al. examined the use of lymphocyte subsets as a potential means to distinguish between severe atopic dermatitis and DOCK8 deficiency [14•]. In two cohorts of children with an established diagnosis of either severe atopic dermatitis or DOCK8 deficiency, quantitative differences in lymphocyte subsets were noted, in particular decreased total $CD3^+$ and $CD4^+$ T cells, decreased naïve $CD8^+$ T cells, and alterations in B cell subsets (increased naïve $CD19^+CD27^-$ B cells and transitional $CD19^+CD24^{hi}CD38^{hi}$ B cells; decreased non-switched $CD19^+CD27^+IgD^+$ memory B cells, and switched $CD19^+CD27^+IgD^-$ memory B cells) [14•]. When all seven parameters were abnormal, the odds ratio in favor of DOCK8 was 26.3 (9.4–73.4). However, focusing on B cell subsets, when all four were abnormal, the odds ratio for DOCK8 deficiency was 33.1 (8.6–127.2) [14•]. Therefore, specific DOCK8 protein flow and detailed lymphocyte subset quantitation have practical value in the diagnosis of DOCK8 deficiency.

X-Linked Lymphoproliferative Syndromes, Types 1 and 2 (SAP and XIAP Deficiencies)

As with DOCK8 deficiency, flow cytometry can be used in the detection of SLAM-associated protein (SAP) in the diagnosis of X-linked lymphoproliferative (XLP) syndrome, type 1. XLP is clinically characterized by hemophagocytic lymphohistiocytosis (HLH), frequently related to Epstein-Barr virus infection, hypogammaglobulinemia, and a propensity to develop lymphomas. This disease is caused by a pathogenic variant in the *SH2D1A* gene encoding the SAP protein. SAP is expressed in T cells, where it plays an important role in T cell interaction with B cells, NK cells, and other T cells. Gifford et al. investigated the utility of SAP protein detection by flow cytometry for diagnosis of XLP [15••]. In 13 of 15 samples harboring known pathogenic *SH2D1A* variants, SAP expression, measured by flow cytometry, was decreased, whereas normal SAP expression was detected in 109 of 122 samples without a variant (sensitivity 87%; specificity 89%) [15••].

Type 2 XLP was initially described in patients who lacked a pathogenic variant in *SH2D1A*, but had an otherwise similar clinical phenotype. The causative variant was described in 2006 as a variant in *BIRC4* (also termed *XIAP*) encoding X-linked inhibitor of apoptosis (XIAP) [16]. While the characteristic clinical feature is also EBV-associated HLH, the clinical phenotype may vary widely and include mononucleosis, splenomegaly, inflammatory bowel disease, uveitis, antibody deficiency, severe Giardia infections, and fistulating skin abscesses [17]. As with DOCK8 and SAP, XIAP levels may be detected via intracellular flow cytometry [17, 18]. Definitive diagnosis requires genetic analysis. Gifford et al. showed sensitivity and specificity of XIAP protein expression as measured by flow cytometry to be 95 and 61%, respectively, and that only one sample out of 60 with normal XIAP expression harbored a pathogenic variant in *XIAP*/*BIRC4* [15••]. As with DOCK8, a missense variant that enables the production of normal protein amounts can generate a false-negative result. This outcome occurred in two patients with missense pathogenic variants and minimally reduced XIAP expression, as measured by flow cytometry [17]. If normal protein levels are present, but clinical suspicion remains high for XIAP, or a genetic variant is identified in the *BIRC4* gene, then a functional assay could be performed to confirm diagnosis. Ammann et al. introduced a novel functional assay measuring the TNF-α response to monocyte stimulation with a lipidated muramyl dipeptide (L18-MDP) [19]. When compared with normal controls or patients with other immunodeficiency conditions, only XIAP-deficient patients demonstrated a reduced TNF-α response to L18-MDP, which is likely due to defective NOD2 signaling [19]. The reduced TNF-α response was noted to be independent of clinical phenotype, amount of XIAP protein, or particular pathogenic variant [19]. Of note, the testing was performed on patients with stable clinical disease, without active immunosuppressant use or evidence of HLH [19]. Thus, flow cytometry-based assessment of SAP and XIAP proteins can be useful in the diagnosis of these specific PIDDs.

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a well-characterized innate immune disorder of neutrophils with impaired NADPH oxidase activity [20], caused by a defect in one of the five protein subunits of the NADPH oxidase complex. Pathogenic variants in *CYBA* (encoding $p22^{phox}$), *NCF1* ($p47^{phox}$), *NCF2* ($p67^{phox}$), and *NCF4* ($p40^{phox}$) result in autosomal recessive (AR) CGD, whereas defects in *CYBB* (encoding $gp91^{phox}$) lead to an X-linked (X-L) inheritance pattern, and typically more severe, accounting for approximately 70% of cases. Clinical manifestations typically include infections with catalase-positive organisms, particularly infections of the skin, soft tissue granulomas, and inflammatory bowel disease. Diagnosis of CGD is usually performed by a flow-based assay using dihydrorhodamine 123 (DHR123), which is a nonfluorescent compound that becomes fluorescent (rhodamine123) during the neutrophil oxidative burst. CGD diagnosis may also be made by the nitroblue tetrazolium (NBT) test, though this has been largely replaced in most clinical laboratories by the DHR test. It is important to note that false-positive results for CGD may be obtained in the

context of complete myeloperoxidase deficiency (cMPO) [21, 22]. Glucose-6-phosphate dehydrogenase (G6PD) deficiency can also cause a false-positive result for CGD; however, these conditions can be ruled out with additional specific testing. Further evaluation of an abnormal DHR result can be performed with a newly developed NADPH oxidase-specific protein flow assay (Abraham et al., personal communication). The NADPH oxidase-specific protein flow assay includes assessment of gp91^{phox}, p47^{phox}, p22^{phox}, and p67^{phox} in granulocytes and monocytes, as well as p40^{phox} in monocytes (Abraham et al., personal communication). The DHR flow cytometry pattern may be useful in identifying the underlying genetic etiology of CGD [23], though this may not hold true in atypical manifestations of the disease. Therefore, genetic testing, correlation with the DHR flow data, NADPH oxidase-specific flow, and clinical phenotype are useful for confirming a diagnosis of CGD.

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) has a prevalence of up to 1:10,000 to 1:50,000 [24] and represents the most common primary immunodeficiency aside from selective IgA deficiency, which is often asymptomatic. CVID is clinically, immunologically, and genetically heterogeneous; however, the common diagnostic features are impaired humoral immunity and susceptibility to infections, autoimmunity, and malignancy [25]. The age of onset is quite variable and can occur during childhood (> 4 years of age) or adulthood [26••]. The clinical spectrum of CVID can range from bronchiectasis, enteropathy, splenomegaly, nodular lymphoid hyperplasia (NLH/LNH), autoimmune cytopenias, organ-specific autoimmunity, and granulomatous disease [26••, 27]. CVID is a diagnosis of exclusion [24], and only one fourth to one third of the patients have an identified monogenic defect. The vast majority of CVID patients likely have a polygenic etiology. Immunological analysis can be useful in the prognosis and classification of CVID patients. Among the immunological studies used for the assessment of CVID patients are lymphocyte subset phenotyping in both B and T cells, B cell functional studies (vaccine antibody response), T cell functional studies, immunoglobulin levels, and other ancillary analyses.

Several classification schema utilizing total B cell counts and B cell subsets have been proposed for CVID, including the Freiberg [28] and the EUROCLASS [29] studies, which correlated B cell subset distribution with clinical phenotype. More recently, three new classifications of CVID have been proposed, including the most recent ICON criteria (ICON classification) [30, 31•, 32••]. The Ameratunga 2013 classification revised the definition of CVID beyond immunoglobulin levels and vaccine antibody response to include B cell subset distribution. This classification scheme also included symptom-related criteria [30]. The ICON criteria offer a broader approach, including asymptomatic diagnosis of CVID based on lab parameters only, as well as incorporation of B cell subsets (memory B cell subsets and CD21^{low} B cells). The ICON criteria do not include use of autoantibodies in CVID diagnosis [32••].

Among the monogenic defects associated with CVID are *TNFRSF13C* (BAFF-R) [33], *TNFRSF13B* (*TACI*) [34–38], *ICOS* [39, 40], *CD19* [41], *CD20* [42], *CD21* [43, 44], and *CD81* [25, 45]. CD27 deficiency causes a combined deficiency with hypogammaglobulinemia and susceptibility to EBV infection [46]. BAFF-R and TACI belong to the tumor necrosis factor (TNF) family of receptors, with BAFF-R binding BAFF exclusively, while TACI can bind both BAFF and APRIL [36, 47]. BAFF-R is required for advancement of B cells beyond the transitional stage of development [33, 47]. *TACI* pathogenic variants have been observed to be associated with autoimmune thrombocytopenia, splenomegaly, and lymphoid hyperplasia [35], with different phenotypes depending on the presence of a homozygous versus heterozygous pathogenic variant, the latter of which displays more autoimmunity [37]. Homozygous *ICOS* variants are associated with impaired T cell-mediated B cell development and differentiation [39]. CD19, CD21, and CD81 form the B cell co-receptor complex, and along with the B cell receptor (BCR) is vital for B cell activation. Pathogenic variants in any of these three components of the BCR complex result in impaired antigenic stimulation [41, 43–45], and reduced class-switched sIgD-CD27^{+} memory B cells, decreased IgG levels, and poor response to polysaccharide vaccination [42]. CD20 is critical for T-independent B cell responses. Given the genetically heterogeneous nature of CVID, broader evaluations of single nucleotide polymorphisms (SNPs) and copy number variations (CNV) have been attempted, the results of which have highlighted the complex and often polygenic nature of this condition [48].

In addition to being at increased risk for autoimmunity and malignancy, a small subset of CVID patients also possess substantial T cell defects leading to a late-onset combined immunodeficiency (LOCID). This subset was further characterized by Malphettes et al., who reported a LOCID diagnostic rate of 8.9% in patients with opportunistic infections and/or a CD4^{+} T cell count $< 200 \times 10^{6}$ cells/L [49]. Patients with LOCID were noted to have an earlier age of diagnosis (31 versus 35 years for CVID), more frequent hospitalization, use of antibiotics, and diagnosis of bronchiectasis, as well as an increased incidence of splenomegaly, granulomatous disease, gastrointestinal disease, and lymphoma [49]. Additionally, they demonstrated lower total lymphocyte count, decreased CD4^{+}CD45RA^{+}CCR7^{+}-naïve T cells, and decreased CD19^{+} B cells [49]. Taking these observations into account might help to more readily distinguish LOCID patients from CVID, afford more tailored therapeutic options,

and lead to improved pathophysiological understanding. The morbidity and mortality of CVID is influenced by the underlying immunological and genetic anomalies in addition to the clinical phenotype [26••, 27, 50, 51].

CVID-Like Conditions

Other genetic defects have been described that are classified in certain databases (OMIM) as being associated with CVID, such as *CTLA-4*, *LRBA*, *NFkB1*, and *NFkB2*; however, it may be more appropriate to refer to these conditions by their specific genetic defects. In these conditions, the clinical and immunological phenotype is quite broad, but usually reflects an underlying immune dysregulation.

Similar to SAP and XIAP, *CTLA4* (cytotoxic T lymphocyte-associated protein 4, CTLA-4) haploinsufficiency with autoimmune infiltration (CHAI) and *LRBA* (lipopolysaccharide-responsive beige-like anchor) deficiency with autoantibodies, regulatory T cell defects, autoimmune infiltration, and enteropathy (LATAIE) syndromes present with similar clinical manifestations, including lymphoproliferation, lymphocytic infiltration of non-lymphoid organs (particularly gastrointestinal, lung, and brain), hypogammaglobulinemia, autoimmune cytopenia, and inflammatory bowel disease [52•, 53]. CTLA-4 is involved in downregulation of the immune response and, thus, is only present on conventional T cells after stimulation (though it is constitutively expressed on regulatory T cells). Prior to expression, it resides in cytoplasmic vesicles which are tended by LRBA. In the absence of LRBA protein, vesicles containing CTLA-4 are rapidly targeted for lysosomal degradation [52•]. LRBA deficiency tends to present earlier in childhood with more complete penetrance and variable severity, whereas CTLA-4 deficiency has a somewhat later age of onset (later childhood to early adulthood) with incomplete penetrance. Studies of family members of a proband with CTLA-4 deficiency have identified the same variant found causative in the proband in 40% of asymptomatic family members [52•].

LRBA deficiency can be assessed by measuring LRBA protein expression by flow cytometry. While most cases of LRBA deficiency have decreased LRBA protein, there are a few examples where LRBA pathogenic variants have demonstrated normal LRBA protein expression. In these cases, functional analysis of LRBA can be very useful in establishing the diagnosis. Hou et al. developed a series of flow cytometry-based assays to help identify and distinguish LRBA and CTLA-4 disease. Memory regulatory T cells, which express the highest amount of CTLA-4, can be used as a cell subset to measure the amount of CTLA-4 protein expression [54••]. CHAI patients demonstrate a fivefold decrease in protein expression compared to healthy controls, while LRBA-deficient patients have even lower amounts [54••]. T cell stimulation results in reduced CTLA-4 protein expression in CHAI patients, but results in a normal response in LRBA deficiency [54••]. Another assay for the specific diagnosis of CHAI includes measuring soluble ligand uptake by CTLA-4 relative to total CTLA-4 protein per cell. CHAI demonstrates significantly reduced amount of ligand uptake relative to healthy controls or LRBA-deficient patients [54••]. For assessment of LRBA deficiency, BafA inhibition of lysosomal degradation can be used to rescue CTLA-4 expression, resulting in increased CTLA-4 protein in stimulated T cells of LRBA-deficient patients compared to healthy controls, or CHAI patients [54••]. CHAI and LATAIE represent PIDDs where disease-specific therapy can be implemented. In these two diseases, treatment with abatacept, a fusion protein of the extracellular CTLA4 domain with the Fc domain of IgG1 (CTLA4-Ig), has shown remarkable benefit [55••].

Evaluation of Innate Immune Deficiencies

The innate immune system represents the initial line of immune defense against invading pathogens. Although nonspecific in nature, it plays a vital role in the early identification of and response to conserved bacterial, mycobacterial, viral, and fungal pathogens. Defects in the innate system cause several PIDDs, with the potential for a severe clinical phenotype [56••, 57••].

Toll-like receptors (TLRs) form the backbone of the innate immune response by recognizing both intracellular and extracellular foreign proteins and nucleic acid motifs. Pathogenic variants in TLRs or their signaling pathways can result in significant clinical disease [56••, 57••]. Interleukin-1 receptor-associated kinase 4 (IRAK-4; *IRAK4*) and myeloid differentiation primary response 88 (MyD88; *MYD88*) are key signaling molecules for most TLR and interleukin-1 receptor families. Pathogenic variants in either gene can render patients subject to life-threatening bacterial infections [58]. TLR3 signals via pathways independent of IRAK-4 and MyD88. Defects in TLR3 can result in an increased risk for herpes simplex virus encephalitis (HSE) due to specific impairment of viral response within the central nervous system [59]. A similar clinical phenotype can manifest due to pathogenic variants in *Unc-93 homologue B1* (*UNC93B1*), which mediates endosomal TLR signaling [60]. Pathogenic variants in several other TLR3-related and unrelated genes share this clinical susceptibility to HSE [56••, 57••, 61].

Laboratory evaluation of TLR defects can be challenging, and Orange et al. have proposed an ELISA-based TLR ligand-induced TNF-α production assay to detect abnormal TLR function [62]. This assay may have utility in the diagnosis of *IRAK4* deficiency, as well as in *IKBKG* (*NEMO*), *I*κ*B*α, *TLR2*, and *TLR4* deficiencies [62]. Subsequently, a flow cytometry assay was introduced to enable a more detailed analysis of the TLR signaling pathways [63].

Alterations in the signal transducer and activator of transcription (STAT) protein family, which are intracellular transcription factors, can also lead to significant abnormalities of the innate immune system. Variants resulting in *STAT1* loss-of-function (LoF) have been associated with severe viral and mycobacterial disease, and can present with severe, life-threatening viral infections due to impaired interferon response [64]. In contrast, a gain-of-function (GoF) phenotype can manifest with chronic mucocutaneous candidiasis (CMC) and autoimmunity in addition to features of immunodeficiency [56••, 57••]. STAT2 deficiency has been associated with a milder susceptibility to viral illnesses than STAT1 LoF, though case reports of fatal disseminated measles have occurred following routine vaccination [65, 66]. *STAT3* genetic variants may be associated with a LoF or GoF phenotype, similar to STAT1 [67]. STAT3 GoF is associated with lymphoproliferation and immunodeficiency, whereas STAT3 LoF is exemplified by autosomal dominant Hyper-IgE syndrome [67]. Determining the function of specific *STAT3* variants requires additional analysis, usually functional in nature, such as phosphorylation kinetics of specific STAT proteins [67]. STAT5b deficiency is the only one among the STAT defects that presents with growth hormone insensitivity in addition to immunodeficiency [56••, 57••]. STAT pathway abnormalities can be assessed in the laboratory using flow-based phosphorylation assays in the presence or absence of specific cytokine stimulants.

Phosphorylation assays have been pivotal in the investigation of intracellular signaling pathway activity and competency. Such assays have been employed to assess the function of tyrosine and serine threonine kinases for phosphorylating intracellular targets [68, 69]. This method involves rapidly fixing cells to preserve accurate protein phosphorylation, labeling phospho-proteins with specific monoclonal antibodies, and also labeling target lymphocyte populations and subsets [68, 69]. This technique has been used in the analysis of TLR signaling as well as specific STAT function [70–73] and MAPK/ERK signaling pathways to identify functional defects of innate immunity [74]. Of note, STAT4 phosphorylation analysis enables the assessment of IL-12 signaling, the impairment of which can manifest as disseminated non-tuberculous mycobacterial (NTM) infections [72]. It is relevant to note that flow-based analysis of STAT phosphorylation is fraught with technical difficulties requiring careful development and validation of the assay to ensure accurate results.

New Technologies: Mass Cytometry

Mass cytometry is a relatively new method of analysis that combines the modalities of flow cytometry and mass spectrometry, while offering increased throughput, and the ability to analyze both protein and nucleic acid targets simultaneously [75, 76, 77••]. By eliminating the difficulties of spectral overlap and limited number of fluorophores in fluorescence-based cytometry, mass cytometry supports the simultaneous detection of a much greater number of cellular targets at a single-cell resolution. Despite these advantages, this technique is still within the research domain and has not been widely utilized in the clinical laboratory due to expense, complexity of data analysis, and relatively lower throughput.

While multi-parameter flow cytometry has become increasingly prevalent, the challenges with spectral overlap can constrain measurement of multiple markers on a single cell. Mass cytometry avoids these difficulties altogether by tagging specific antibodies with a unique lanthanide epitope, which can then be measured via a mass spectrometry detector. In this manner, the number of simultaneously identifiable targets can be increased to over 40 cellular components (protein and/or RNA) simultaneously [76, 77••]. Mass cytometry allows for both liquid- and solid-phase analyses, and despite certain limitations of cost and throughput, it affords a valuable tool for research and discovery in PIDD.

Practical applications of mass cytometry in this field include mechanistic studies of CVID [78]. Cols et al. used mass cytometry to elaborate on previous transcriptome profiling, and identified a distinct signature of increased type 3 lymphocytes producing interferon-γ in a subset of CVID patients with inflammatory complications [78]. The breadth and depth of cellular analysis available through mass cytometry is likely to enhance our knowledge of the pathophysiology of many PIDDS, and will likely gain a strong foothold within the clinical environment.

Application of Flow Cytometry and Molecular Technologies in Newborn Screening for Severe Combined Immunodeficiency and Related Disorders

Newborn screening is a rapidly expanding public health program used to screen asymptomatic infants for potentially serious and actionable genetic disorders. Since 2008, newborn screening for severe combined immunodeficiency (SCID) has been used to identify infants with life-threatening T cell lymphopenia, and other immunological defects using a molecular marker called TREC (T cell receptor excision circle) [79, 80••]. The sensitivity of the newborn screen TREC assay was found to be 100% in a systematic review of 3.15 million newborns, with 142 diagnosed SCID cases [81•]. In a prospective cohort, the positive predictive value was 0.8 to 11.2% for SCID and 18.3–81.0 for T cell lymphopenia [81•]. The differential for an abnormally low TREC result is broad and includes false positives, prematurity, illness, identifiable genetic syndrome (trisomy 21, trisomy 18, Jacobsen syndrome, etc.), and multiple other immunodeficiency diseases, including DiGeorge and CHARGE, among others. All abnormal newborn screen SCID results are evaluated further by flow cytometry for lymphocyte subsets and distribution of

naïve and memory T cells. Additional immunological investigation and genetic testing is often warranted, depending on the result of these tests and clinical phenotype. An example of evaluation of an abnormal newborn screen SCID result has been reported by Mauracher [82•]. Among the flow cytometry tests used to follow up abnormal TREC results, the quantitation of T, B, and NK cells permits a rapid genetic triage for SCID defects [80••, 83].

The newborn screen TREC results can be validated independently in the clinical laboratory using a TREC assay performed by real-time PCR, which normalizes TREC copies relative to the patient's $CD3^+$ T cell count (Abraham et al., personal communication, 2017). TREC analysis may also be performed in the clinical laboratory using a newer technique of digital droplet PCR (ddPCR) [84••]. The ddPCR method [85] offers improved efficiency and throughput compared to real-time quantitative PCR. With this method, each sample is divided into thousands of equally sized droplets or partitions, and PCR is subsequently performed within each, individual droplet with results then assessed across the entire field (based on the Poisson distribution) to provide an absolute concentration of the DNA target. This method has been applied to the diagnosis of 22q11.2 deletion syndrome [86••] and has also been used in TREC determination within the newborn screening program [84••]. TREC-based newborn screening using ddPCR demonstrated a clinical sensitivity of 88.9% and a specificity of 100%, with similar costs to quantitative PCR [84••].

While some conditions, such as 22q11.2 deletion syndrome, are not yet specifically present on newborn screening algorithms, they are included in the differential for abnormal TREC results in infants with T cell lymphopenia (TCL). It has been estimated that 29% of cases of non-SCID lymphopenia detected on newborn screening is due to 22q11.2 syndrome [87••]. Diagnosis of this condition is currently determined by fluorescence in situ hybridization or genetic testing, which can be cumbersome and expensive. Kobrynski et al. have developed a MALDI-TOF mass spectrometry assay to directly assess for 22q deletion using a multiplex competitive PCR assay on dried blood spots [87••]. Though still in a nascent phase, this technique may help to expand the capability of a more rapid, specific newborn screening algorithm. MALDI-TOF mass spectrometry assay has also been used to diagnose adenosine-deaminase deficiency from dried blood spots at a minimal cost [88].

Characterization of T cell receptor (TCR) diversity via molecular techniques can be a useful tool in analyzing SCID cases and may afford some prognostic implications [89]. TCR repertoire diversity can be assessed by fragment length analysis called spectratyping, and can be valuable in both diagnosis and monitoring of patients with T cell defects [89, 90•, 91••].

Large regions of the globe do not have access to newborn screening programs, and in such cases, diagnosis of SCID is frequently delayed. In a family perspective survey, it was observed that neonatal diagnosis of SCID resulted in better survival, demonstrating the practical utility of universal newborn screening [92]. A study based in China and Southeast Asia found that a family history of early infant death was associated with an earlier age at symptom presentation and clinical diagnosis, but did not afford a shorter time to diagnosis, in a group of 147 patients, including 94 patients with a genetic diagnosis of SCID [93••]. Though diagnosis of these infants was at a median of 4 months, it was still after the 3.5-month recommended hematopoietic cell transplant (HCT) goal for SCID to promote the best survival outcomes [94]. In countries and areas without newborn screening, the use of the absolute lymphocyte count from complete blood count with differential along with the family history, and history or presence of worrisome infections (BCG, candidiasis) may offer early awareness of an underlying cellular immunodeficiency [93••].

These clinical examples offer perspective on the utility of flow cytometry assays and non-genetic molecular assays for the diagnosis, classification, prognosis, and monitoring of PIDDs.

Genetics/Genomics

Rapid improvements in technology and the increasing throughput of in-depth genetic analysis have fostered a relative explosion in knowledge and understanding of PIDDs. The identification of genetic causes of PIDDs has increased from less than 50 in the early 1990s to over 300 at the writing of this article [56••, 57••, 95•]. The advent of next-generation sequencing (NGS) [96] has enabled the development of targeted gene panels, whole exome sequencing, and even whole genome sequencing as additional tools to help identify underlying defects. With these new technologies have come the challenges of data processing and validation techniques to help sort through increasingly large datasets generated by these high-throughput techniques. Given the diverse phenotype-to-genotypic relationship of PIDDs, genetic analysis has proven quite useful in solving previously undiagnosed cases (Fig. 1b).

Genetic analysis plays a vital role in diagnosis of newborn screen SCID, including targeted gene sequencing (TGS), whole exome sequencing (WES), and chromosomal studies. Practical applications of genetic testing have been described in several studies [97•, 98]. Genetic diagnosis in this context enables selection of appropriate therapy, which is critical to overall outcomes in these patients (Yamazaki Y and Zhang K et al., manuscript submitted, 2018).

Targeted Gene Sequencing

TGS panels are customizable sets of genetic targets covering a known group of disease-causing genes. These panels may include exons as well as introns or regulatory regions of known clinical significance, which are often excluded from whole exome sequencing, or incompletely evaluated, either due to low coverage or high homology. As the number of genes is limited, the coverage depth of each area is much improved [99], though false negatives can still occur [100]. The cost of TGS is favorable compared to broader sequencing techniques, and the data generated is more manageable for subsequent analysis [101•]. The overall diagnostic efficiency for TGS panels in patients with PIDD lacking in a genetic diagnosis has been reported to be 15–25% for panels including approximately 160–170 genes [100, 102, 103]. As previously mentioned, TGS has already shown utility in the genetic diagnosis of newborn screen SCID patients [98]. While useful in terms of cost and inclusion of known pathologic variants, a limitation of TGS is that the panel design is pre-set and thus may miss other pertinent genes or identification of novel variants, resulting in false-negative calls. Mousallem et al. have chronicled several examples of pathogenic variants that were missed on initial genetic analysis, but subsequently discovered on whole genome sequencing (WGS) [104•]. In one example, two female siblings with a clinical diagnosis of CGD based upon a clinical history and abnormal DHR oxidative burst analysis had negative TGS for this condition. Subsequent whole genome sequencing identified a previously reported homozygous *NCF1* c.579G>A pathogenic variant, resulting in a premature stop codon [104•]. Many commercial gene panels offer only the common exon 2 ΔGT deletion for the *NCF1* gene resulting in false-negative results when other pathogenic *NCF1* variants are present [104•]. In general, it is important to note that genetic tests will only detect variants in genes/regions included in design of the test. Additionally, given that new genes are being associated with PIDD phenotypes on a regular basis, it can be difficult to keep TGS panels current.

Whole Exome Sequencing

WES covers over 90% of the human exome and the majority (85%) of known pathogenic variants with respect to PIDDs [99]. Stray-Pedersen et al. employed WES to re-assess the molecular diagnosis of 278 PIDD patients and their families [105••]. The diagnostic yield of WES was greatest for patients with SCID, bone marrow failure, and syndromic PIDD, with an overall average of 39.6% [105••]. Other studies have reported a lower diagnostic efficiency of WES at 20% [99]. However, WES was sensitive to detect low-level mosaicism in several probands/families as well as probable somatic relevant mosaicism in three families with pathogenic variants in *IKBKG*, *FANCA*, and *IL7R* [105••]. It additionally changed the clinically derived diagnosis in 55% of families [105••]. A role for WES may also be present for investigating the presentation of unusually severe infections in previously healthy children. Asgari et al. identified novel pathogenic variants using WES in known PIDD genes in two out of eight patients with fatal community-acquired *Pseudomonas* sepsis, suggesting the utility of WES in such cases [106]. While assessment of copy number variation (CNV) is not typically considered a strength of exome sequencing, specific read-depth analysis has been successfully employed to assess CNV in exome datasets [107•]. The potential limitations of WES include the need to preferentially enhance the exome from the remainder of the genome [99], and enrichment is not equally consistent across all regions of DNA due to variations in guanine-cytosine content, sequences with high homology, and nucleotide repeats [95•]. PCR amplification, which is sometimes required during sequence library preparation, can introduce further bias in WES analysis [95•]. Finally, due to the increased volume of genetic material assessed, coverage of any one specific area is typically reduced, which may result false-negative sequence results [99]. Despite wide genomic coverage, including the majority of recognized, PIDD-causing variants, WES only covers about 1–2% of the total genome and typically does not cover deep intronic or regulatory regions that may involve disease processes.

Whole Genome Sequencing

WGS covers the entire span of human DNA, including both coding and noncoding regions. It typically offers more homogenous genomic coverage as it does not require prior PCR or hybridization enrichment [95•, 99]. As a result, copy number and structural variants may be assessed with less potential for bias than with TGS or WES [99, 107•]. Though the coverage is more homogenous, the depth of coverage is the least of the three sequencing techniques. The lack of coverage depth in whole genome sequencing can cause false-negative calls, as observed by van Schouwenberg when heterogeneous variants in *TNFRSF13C* (BAFF-R) were found on Sanger sequencing after being missed on WGS [108••]. Despite the potential for false-negative results, WGS has been successfully employed to render a diagnosis within genes previously assessed by either targeted panels or specific genetic screening as noted above with the *NCF1* variant. Another example includes a false-negative result for a *DCLRE1C* (Artemis) variant, which was missed by gene sequencing [104•]. Unlike more limited testing, WGS has the advantage of examining the entirety of the human genome for potentially causative variants, which facilitates discovery of new genes associated with PIDD. While the majority of disease-causing variants for PIDD occur within the exome, disease-causing variants can occur in non-exonic DNA. These include pathogenic variants

in noncoding RNA genes associated with cartilage-hair hypoplasia (CHH) and Roifman syndrome (RFMN), as well as X-linked reticular pigmentary disorder, which is caused by intronic variants [95•]. van Schouwenberg et al. combined whole genome sequencing with transcriptome profiling and structural mapping to identify a novel heterozygous *LRBA* variant predicted to affect protein stability, as well as novel heterozygous variants in *BTK* and *BLK* [108••]. Data processing algorithms and subsequent analysis procedures for WGS, as well as WES, are critical given the substantial size of generated datasets.

Transcriptome Analysis

The addition of transcriptome profiling has been found beneficial in teasing out disease-causing variants from the large dataset generated by both WES and WGS. Khan et al. utilized WES followed by whole blood RNA sequencing to help narrow a causative variant from 24 candidates to a single, novel variant in *DOCK8* leading to nonfunctional DOCK8 transcripts of varying lengths [109•]. Given that the majority of transcribed RNA is noncoding in nature, transcriptome analysis could afford another layer of high-throughput screening for PIDDs (Cousin et al., manuscript submitted, 2017). As previously mentioned, transcriptome profiling in conjunction with WGS has been beneficial in identifying novel variants in *LRBA*, as well as in characterizing the general enrichment in BCR pathway signaling defects in CVID patients [108••].

Other Considerations in Genetic Testing

Despite their proven clinical utility, NGS technologies have some limitations, which should be considered when utilizing these tools [110]. Small insertion or deletions, repetitive sequences, and areas with high sequence homology or low complexity may be missed. Additionally, CNV, duplications, and large structural variants can be problematic, though additional analysis may help overcome these limitations [105••]. NGS offers the capability to detect CNV and structural rearrangements [110], though often confirmatory methods, such as Sanger sequencing or multiplex ligation-dependent probe amplification (MLPA), may be required to investigate these areas further. For example, variants in the *CFHR* genes can cause a variety of clinical disorders, including atypical hemolytic uremic syndrome, glomerulopathies, nephropathy, and systemic lupus erythematosus. Genetic analysis is complicated by significant sequence homology between the five *CFHR* genes [111], making detection challenging via NGS. Supplemental Sanger sequencing can help to circumvent this challenge.

Data volumes from high-throughput molecular sequencing are quite large and require significant post hoc analysis to identify the disease-causing variant(s). Incidental findings are important to consider as are variants of unknown significance (VUS) and variants in genes of unknown significance (GUS). It is estimated that each individual possesses approximately 20,000 coding variants detectable by high-throughput screening [112••]. Some groups have attempted to address the challenge of sorting through such high numbers of candidate variants by computational methods, such as using biological clustering to predict genes that are more likely related to PIDDs. Using such a method, Itan and Casanova were able to identify and validate a candidate list of 3110 genes potentially related to PIDDs [112••]. Although still a daunting number, it represents an 84% decrease in total number of variants and might enable more focused analysis of the remaining candidate variants. Prior investigation has shown that up to 27% of literature-reported pathogenic variants were later found to have been mis-annotated or common polymorphisms [110]. It is also worth mentioning that, in the majority of cases, a genetic diagnosis will not be realized with high-throughput genetic analysis. Therefore, for these instances, regular reassessment of patient genetic data may be worthwhile. Given the number of causative variants discovered annually, revisiting older datasets could be of potential benefit in ultimately achieving a molecular diagnosis for some patients [110]. Finally, as with many uses of broad-spectrum genetic screening, unrelated incidental findings may be identified that have a known health effect, impact other family members, and/or implicate serious disease. The ACMGG recommends that a subset of actionable incidental findings be reported to the family or individual pursuing the test [110, 113]. In addition, there is a potential to identify VUS and GUS. Appropriate counseling prior to testing is imperative to inform patients of the possibility of incidental and/or uncertain findings.

Other useful genetic techniques have also been developed to help probe the underpinnings of immunodeficiency. As previously mentioned, *STAT1* variants can present with either a loss-of-function or a gain-of-function phenotype with mycobacterial susceptibility or chronic mucocutaneous candidiasis, respectively, which is not predicted based upon the location of the variant. As variants typically occur within the coiled-coil or DNA-binding domains, Kagawa et al. employed an alanine-scanning mutagenesis assay in which alanine residues were methodically substituted for amino acids within these two domains of STAT1 [114••]. After screening 342 different alanine variants, 100% of the known loss-of-function and 78.1% of the gain-of-function variants in *STAT1* were correctly identified [114••]. Such a technique may be a useful alternative to or companion of computational techniques to help characterize unknown variants in the future. Grodecka et al. have identified mRNA splicing defects in 21.7% of PIDD-related variants using a mini-gene splicing assay [115••]. Such a method may augment already available splicing prediction tools [116•].

As previously mentioned, pseudogenes and CNV can pose difficulties with targeted gene panels as well as WES.*NCF1*,

which encodes the p47[phox] subunit of the NADPH complex, is associated with two pseudogenes. Cross-over from these pseudogenes can lead to autosomal recessive CGD [117•]. In addition to being detected on WGS [104•], multiplex ligation-dependent probe amplification (MLPA) has been used to successfully examine copy number and unequal cross-over from *NCF1* pseudogenes in a cohort of p47[phox]-deficient patients [117•]. In an alternative method, digital droplet PCR was employed to assess the ratio of GTGT alleles to total *NCF1* alleles to help distinguish normal, carrier, and affected patients with autosomal recessive p47[phox] CGD [118•].

CNV has also been assessed in CVID, among other disorders. Orange et al. performed a genome-wide association study (GWAS) assessing single nucleotide polymorphisms (SNPs) and copy number variation (CNV) among patients with CVID [48]. This analysis highlights the polygenic nature of CVID compared to the monogenic PIDDs. Using a support vector machine (SVM) algorithm, they were able to develop and validate a group of 1000 SNPs that, taken together, were able to accurately distinguish an independent cohort of CVID versus control patients [48]. Other analyses of CNV in CVID found higher copy number variation among patients with CVID that did not correlate with clinical phenotype [119•].

Conclusion

The field of inborn errors of human immunity (PIDDs) continues to expand in terms of knowledge and understanding of the molecular pathophysiology of disease. As elaborated in this review, there are many diagnostic tools available for evaluation of PIDD (Table 1), but these need to be used with appropriate understanding to ensure relevant employment, which facilitates a cost-effective, efficient, and timely diagnosis. The specific approach used will vary depending upon the clinical and immunological phenotype, and underlying disease (Fig. 1a). The increasingly widespread use of newborn screening for SCID/T cell lymphopenia coupled with appropriate follow-up testing offers the potential of pre-symptomatic diagnosis, with much improved survival rates of properly identified and treated patients. More high-throughput molecular and genetic techniques, such as mass cytometry and genetic testing (Fig. 1b), will enable broader testing, but will require increased computational assessment necessitating high-quality algorithmic methods. While some techniques are readily available in a clinical setting, others remain more research-based. The efficient and thoughtful use of these different methodologies, always with the clinical phenotype and family history in mind, may help to afford more timely and accurate diagnosis of primary immunodeficiency disorders.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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