



A Critical Review on the Standardization and Quality Assessment of Nonfunctional Laboratory Tests Frequently Used to Identify Inborn Errors of Immunity

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Inborn errors of immunity (IEI), which were previously termed primary immunodeficiency diseases, represent a large and growing heterogeneous group of diseases that are mostly monogenic. In addition to increased susceptibility to infections, other clinical phenotypes have recently been associated with IEI, such as autoimmune disorders, severe allergies, autoinflammatory disorders, benign lymphoproliferative diseases, and malignant manifestations. The IUIS 2019 classification comprises 430 distinct defects that, although rare individually, represent a group affecting a significant number of patients, with an overall prevalence of 1:1,200-2,000 in the general population. Early IEI diagnosis is critical for appropriate therapy and genetic counseling, however, this process is deeply dependent on accurate laboratory tests. Despite the striking importance of laboratory data for clinical immunologists, several IEI-relevant immunoassays still lack standardization, including standardized protocols, reference materials, and external quality assessment programs. Moreover, well-established reference values mostly remain to be determined, especially for early ages, when the most severe conditions manifest and diagnosis is critical for patient survival. In this article, we intend to approach the issue of standardization and quality control of the nonfunctional diagnostic tests used for IEI, focusing on those frequently utilized in clinical practice. Herein, we will focus on discussing the issues of nonfunctional immunoassays (flow cytometry, enzyme-linked immunosorbent assays, and turbidimetry/nephelometry, among others), as defined by the pure quantification of proteins or cell subsets without cell activation or cell culture-based methods.

Keywords: inborn errors of immunity, primary immunodeficiencies, quality assessment (QAS), standardization, reference range

INTRODUCTION

Primary immunodeficiency diseases (PID) constitute a large and fast-growing heterogeneous group of genetic diseases, mostly (but not exclusively) caused by loss or gain of function germline mutations. Although PID are classically manifested as increased susceptibility to infections, recently, an increasing variety of autoimmune, autoinflammatory, allergic, and malignant phenotypes has also been recognized (1). This comprehensive concept was closely accompanied by a broader pathophysiological understanding of such disorders, which are now grouped in the category of inborn errors of immunity (IEI) (2). Despite individual rarity, IEI collectively represent a significant proportion of patients, with an estimated overall prevalence of 1:1,200-2,000 (3, 4). They now comprise 406 distinct disorders with 430 different gene defects subdivided into categories listed in the 2019 International Union of Immunological Societies (IUIS) classification (5, 6), approximately two-thirds of which were recognized in the past decade (**Table 1**). As evidence of dynamic development in the area, 26 additional monogenic gene defects have recently been reported and may soon be included in the IEI portfolio (8).

Early specific diagnosis of IEI patients is deeply dependent on accurate lab tests, and is pivotal for targeted therapy and appropriate patient and familial genetic counseling (2). In this context, the laboratory serves as the primary source of diagnostic information used to define the underlying immunologic defect (9). Clinically guided IEI laboratory investigations may follow three main consecutive steps: screening, advanced testing, and molecular confirmation (**Table 2**). Flow cytometry assays and molecular analyses are currently the most relevant methodological platforms in the area. Moreover, functional tests are critical for identifying particular IEI. Some assays are simple and disseminated worldwide, while others are only available in specific research centers, creating an obstacle for knowledge spread in the area.

Despite the striking importance of laboratory data for clinical immunologists, several IEI relevant immunoassays still lack standardization, including standardized protocols, reference materials, and external quality assessment programs. Moreover, well-established reference values mostly remain to be determined, especially for early ages, when the most severe conditions manifest and diagnosis is critical for patient survival (10). Compared to biochemical tests, standardization and quality controls in immunoassays are rudimentary, partially due to the particular complexity of analytes. Immunoassays usually assess heterogeneous molecules, such as serum polyclonal antibodies, that share common characteristics, but are in fact distinct analytes with individual features.

The above-described issues reinforce the necessity of a parallel healthy control blood sample in some IEI diagnostic-driven nonfunctional immunoassays, assuming a high number of uncontrolled variables. This is particularly problematic in young patients whose blood is usually compared with adult control samples. Although challenging, tests for the identification of IEI need better standardization to improve the diagnostic accuracy. Such a hard task has precedents in other areas, such as the prothrombin activity assay, which, in the near past, was totally

uncontrolled and is currently standardized within an international normalized ratio.

In this article, we will approach the issues of methodological standardization (including the definition of reference ranges) and quality control programs for nonfunctional tests used to identify IEI, focusing on those frequently utilized in clinical practice. We expect to not only contribute to critical lab result interpretation in bedside clinical evaluations, but also encourage clinical pathologists and researchers to improve the accuracy, reproducibility, and international harmonization of tests relevant to IEI diagnoses. Herein, we will focus on listing all papers addressing standardization and quality assessment programs and discussing the issues of nonfunctional immunoassays (flow cytometry, enzyme-linked immunosorbent assay, and turbidimetry/nephelometry, among others), as defined by the pure quantification of immunological critical molecules or cell subsets without the involvement of cell activation assays or cell culture-based methods.

Single-analyte quantification and flow cytometry-based assessments of the cell surface and intracellular protein expression will be considered nonfunctional tests in our paper. This category consists of both screening (e.g., immunoglobulin serum levels, specific serologies, T cell receptor excision circle quantification, etc.) and advanced tests (e.g., immunophenotyping panels, specific surface, and intracellular protein expression, among others). Cell activation and cell culture-based assays are considered “functional tests” and will not be approached here.

STUDY METHOD

A broad search of the Medline/Pubmed, Google Scholar and Scielo databases was performed using the terms “reference range”, “standardization”, “quality assessment”, “quality control” and “QAS” crossed with all captions representing each IEI subarea below: “predominantly antibody deficiencies”, “IgG/IgM/IgA serum levels”, and “B/T cell immunophenotyping”, among others. The nonsystematic review included every paper approaching any methodological standardization and quality control programs.

REGULATORY AGENCIES AND LAB CERTIFICATION

Current regulation policies demand analytical validity reviews of great depth and scope for any newly developed test system prior to marketing, and, therefore, prior to use with patient specimens in the clinical diagnosis or treatment context. This process is usually performed and regulated by different national agencies (e.g., Food and Drug Administration, European Medicines Agency, Brazilian Health Regulatory Agency, etc.) hence, its validity is specific to the home country, although some nations eventually adopt foreign reviews. Safety and effectiveness assessments of the novel test system may also include the accuracy with which the test identifies, measures, or predicts the presence or absence of a clinical condition in a patient,

TABLE 1 | IEI categories and clinical prototypes according to the 2019 IUIS update of the phenotypical classification (6).

Category	Clinical phenotypes (n)	Clinical prototypes	Causative genes (n)	% of total IEI (7)
Immunodeficiencies affecting cellular and humoral immunity	58	SCID	59	7
Combined immunodeficiencies with associated or syndromic features	68	Wiskott Aldrich syndrome, DiGeorge syndrome, Bloom syndrome, ataxia telangiectasia, hyper-IgE syndrome	63	11
Predominantly antibody deficiencies	48	Agammaglobulinemia, CVID	40	57
Diseases of immune dysregulation	46	HLH, ALPS, IPEX, APECED	45	6
Congenital defects of phagocyte number or function	41	CGD, LAD	41	8
Defects in intrinsic and innate immunity	64	CMC, MSMD, recurrent HSE	67	2
Autoinflammatory disorders	43	FMF, CAPS, TRAPS, MVKD, PAPA syndrome, type 1 interferonopathies	42	3
Complement deficiencies	27	Complement components deficiencies, hereditary angioedema	33	2
Bone marrow failure	8	Fanconi anemia, dyskeratosis congenita	40	3

The total numbers of clinical phenotypes and causative genes are also represented for each category. Of note, these two variables are not always identical due to the presence of different clinical phenotypes caused by a single gene and vice versa. The frequencies of each representative category within the total number of IEI patients according to the Latin American Society of Immunodeficiencies (7) are also depicted.

ALPS, autoimmune lymphoproliferative syndrome; APECED, autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy; CAPS, cryopyrin-associated periodic syndrome; CGD, chronic granulomatous disease; CMC, chronic mucocutaneous candidiasis; CVID, common variable immunodeficiency; FMF, familial Mediterranean fever; HLH, hemophagocytic lymphohistiocytosis; HSE, Herpes simplex encephalitis; IEI, inborn errors of immunity; IPEX, immune dysregulation, polyendocrinopathy, enteropathy X-linked; IUIS, International Union of Immunology Societies; LAD, leukocyte adhesion deficiency; MSMD, Mendelian susceptibility to mycobacterial disease; MVKD, mevalonate kinase deficiency; PAPA, pyogenic sterile arthritis, pyoderma gangrenosum, acne; SCID, severe combined immunodeficiency; TRAPS, TNF receptor-associated periodic syndrome.

constituting a process usually called clinical validity testing. In summary, regulatory agencies ensure that new devices intended for the diagnosis, treatment, or prevention of disease are safe and effective.

On the other hand, quality assessment programs are designed to regulate laboratories that perform testing on patient specimens to ensure accurate and reliable test results. Programs are usually based on regular routine surveys that certify participant labs with governmental or non-governmental institution approval [e.g., Clinical Laboratory Improvement Amendments (CLIA), College of American Pathologists (CAP), Brazilian Clinical Laboratory Accreditation Program, etc.]. Ultimately, the institutions assess the performance characteristics of a test to describe the quality of patient test results, including analyses of accuracy, precision, analytical sensitivity, analytical specificity, reportable range, reference interval, and any other performance characteristics required by the test system in the laboratory that intends to use it. In addition, regulatory requirements vary according to the equipment used and type of test performed: the more complex the test is to perform, the more stringent the requirements. Therefore this analytical validation is limited to the specific conditions, staff, equipment and patient population of the particular laboratory, so the findings of these laboratory-specific analytical validations are not meaningful outside of the laboratory that performed the analysis.

Thus, the two regulatory schemes described above are different in focus, scope and purpose, but they are intended to be complementary. Of note, especially in the United States, when a laboratory develops a test system such as an in-house laboratory-developed test (LDT) without receiving FDA clearance or approval, CLIA rules prohibit the release of any test results prior to laboratory establishment of certain performance characteristics related to analytical validity for the use of that test system in the laboratory's own environment.

In summary, any novel diagnostic system or device requires strictly addressing the following parameters as they apply to regulatory agency approval: accuracy, trueness, precision, reproducibility, robustness, linearity, reportable range, reference range, interfering substances, analytic sensitivity/specificity, limit of detection/quantification, and clinical sensitivity/specificity (11).

PREDOMINANTLY ANTIBODY DEFICIENCY

Predominantly antibody deficiency (PAD) encompasses the most frequent IEI reported in numerous series worldwide (Table 1), representing 60-80% of IEI identified in adults (12). Screening tests include immunoglobulin serum levels (IgG, IgM and IgA), antibody responses to both protein and polysaccharide vaccine antigens, and total circulating mature B cell numbers (CD19⁺ or CD20⁺) (Table 2). B cell immunophenotyping and rarely ordered IgG subclass serum levels should be postponed until the second step (13, 14).

IgG (and Subclasses), IgM, and IgA Serum Levels

Serum IgG, IgA and IgM levels are the most important screening tests for the initial assessment of humoral immunodeficiencies and are usually evaluated by nephelometry or turbidimetry, which provide good correlation indices and fast and highly reproducible results for quantification in serum and other fluids (e.g., cerebrospinal fluid).

Most laboratories have reference values of these parameters for all age groups, which may vary according to different ethnic groups and across countries (15) and are accredited and highly controlled by CAP. Well-established immunoglobulin and IgG subclass levels within two standard deviations (SD) of the mean

TABLE 2 | Suggested IEI clinically guided laboratory investigation guidelines, according to three proposed main consecutive steps: screening, advanced tests, and molecular confirmation.**Screening**

Cell blood count and peripheral smear

Serum immunoglobulins (IgG, IgA, IgM and total IgE)

Vaccine response against polysaccharide (e.g.: *Streptococcus pneumoniae*) and protein antigens (e.g.: tetanus toxoid); spontaneous specific antibodies (anti-blood group Abs, isohemagglutinins)

Peripheral blood basic immunophenotyping: CD3, CD4, CD8, CD19, and CD16/CD56

Complement system assessment: CH50 and AH50

Phagocyte oxidative burst: dihydrorhodamine oxidation assay

TREC copies

Advanced tests

Predominantly antibody deficiencies

IgG subclasses

Vaccine response against neoantigens (bacteriophage, *Salmonella typhi* capsular polysaccharide vaccine)

B cell immunophenotyping

Intracellular protein expression – BTK

Combined immunodeficiencies affecting cellular and humoral immunity

Chromosomal instability

T cell immunophenotyping (flow cytometry)

CD40/CD40L binding assay

Cell surface protein expression – CD132 (IL-2R γ), CD127 (IL-7R α), MHC I and II

Intracellular protein expression – WASP (Wiskott-Aldrich protein)

Lymphoproliferation in response to mitogens, alloantigens and recall antigens

TCR repertoire analysis: TCR-CDR3 spectratyping and flow cytometry-based TCR V β frequency

Adenosine deaminase and PNP activity

In vitro cytokine production in cell culture supernatant

NK cytotoxic activity assay and CD107a degranulation

Diseases of immune dysregulation

Soluble CD25

Intracellular protein expression (PRF1, SAP/SH2DIA, XIAP)

Double negative TCR α / β circulating T cells

Lymphocyte apoptosis assay

Soluble mediators: IL-10, IL-18, soluble FASL and vitamin B12

T regulatory cells (CD4+/CD25+/CD127low/Foxp3+) number and function

STAT1 phosphorylation assay

CTLA-4 functional testing

Defects in intrinsic and innate immunity

IL-12/IFN γ axis functional assayIntracellular protein expression: IFN γ -R1 and IFN γ -R2

Cell surface protein expression: CD18, CD11a/CD11b/CD11c, CD15

I κ B α degradation

TLR functional assays (CD62L shedding)

Autoinflammatory disorders

Type 1 interferon signature

Serum IgD

Urinary mevalonic acid

Complement deficiencies

Specific complement components

Genetic and molecular tests

Karyotype, FISH, MLPA, copy number variation analysis

Specific single gene-sequencing (Sanger)

Next-generation sequencing (panels, whole-exome or genome sequencing)

A list of the main nonfunctional IEI diagnostic tests is provided and should be individually considered according to the stage of investigation.

AH50, total hemolytic complement (alternative pathway); ALPS, autoimmune lymphoproliferative syndrome; CH50, total hemolytic complement (classic pathway); FISH, fluorescence in situ hybridization; IEI, inborn errors of immunity; MLPA, multiplex ligation-dependent probe amplification; TCR, T cell receptor; TLR, Toll-like receptors; TREC, T cell receptor excision circle.

in age-matched controls are considered normal. In clinical practice, two distinct scenarios must always be investigated: i) IgG levels below 400 mg/dL in school children, adolescents, or adults; and ii) serum levels clearly below the age-adjusted reference range (95% CI) in infants or small children (16). Another aspect to be considered is that serum IgG levels in the initial months of life may be masked by maternal IgG transplacental transference. Therefore, a new assessment after six months of life, by which point maternal IgG has already been degraded, is mandatory (17).

It is largely established that in selective IgA deficiency (SIgAD), which is the most common pediatric antibody

deficiency with incidence rates varying between 1:143 and 1:18,500 (18), the serum concentration is always less than 7 mg/dL associated with normal serum IgG and IgM levels. As IgA only reaches adult levels later in life, and SIgAD diagnosis can only be confirmed after four years of age (19).

IgG subclass ordering has restricted utility, and is thus not a consensus for IEI diagnosis, although it can be particularly useful in SIgAD associated with recurrent sinopulmonary infections (20–39).

IgD

Serum IgD levels have usually been assessed by ELISA, with reports of a wide range among healthy individuals (0.10 to 213

µg/ml). Serum IgD concentrations have been shown to increase over childhood and decrease with age, but no normality range has been well-established for different age groups (40, 41).

IgD measurement is not usually included in a standard antibody evaluation; however, this analyte assessment is useful if there is a clinical suspicion of mevalonate kinase deficiency (MKD). In this monogenic autoinflammatory disease (MVK), serum polyclonal IgD concentrations are elevated, with a median of approximately 400 U/mL (1 U = 1.41 µg/mL). MKD is also called hyperimmunoglobulinemia D and periodic fever syndrome or hyper-IgD syndrome (HIDS), although the reason for the increased IgD concentrations and their role in pathogenesis have not yet been fully clarified (42, 43).

Total IgE

Serum IgE levels are usually assessed by ELISA or fluorescent solid-phase immunoassay, however there are no well-established serum IgE reference values for different age groups, especially for healthy infants and children (44–46). IgE serum levels between 100 and 200 kU/L (1 U/L = 2,4 ng/ml) are considered normal for healthy adults (45). Longitudinal studies in “normal” children have demonstrated that IgE levels tend to progressively increase in the first decade of life, with large variability in early first years, followed by plateauing at age 10–13 years, and decreasing slightly in the following years (44).

Allergic disorders are the most frequent cause of high IgE levels, although parasitic infestations may also be relevant conditions in tropical areas (47–49). Regarding IEI, elevated serum IgE levels are associated with several diseases, such as: i) hyper-IgE syndrome (loss-of-function *STAT3* mutation); ii) Deducator of CytoKinesis 8 (DOCK8) deficiency; iii) IPEX – Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (*FOXP3*); iv) Wiskott-Aldrich syndrome (*WAS*); v) Phosphoglucomutase 3 (*PGM3*) deficiency; vi) Comèl-Netherton syndrome (*SPINK5*); and vii) Loey-Dietz syndrome (*TGFBR1*). Of interest, all of these conditions present severe allergic dermatitis (50–52). Elevated IgE levels also represent a characteristic finding in Omenn syndrome (*RAG1*, *RAG2*, *DCLRE1C* or *IL7R*), which is an extremely severe condition seen in some infants with severe combined immunodeficiency (SCID) (53, 54). Very high concentrations of IgE — above 1,000–2,000 kU/L at an early age — should direct attention to an IEI.

On the other hand, IgE deficiency (<2.5 kU/L), which has been considered without clinical consequences for decades, has recently been associated with higher rates and risks for the development of malignancies (55). IgE deficiency is also seen in some IEI, such as ataxia-telangiectasia (*ATM*), as well as in some patients with common variable immunodeficiency (CVID), SIgAD or IgG subclass deficiencies (56–58). Down syndrome patients usually present low total and specific IgE concentrations, even those presenting chronic or recurrent respiratory manifestations (59).

Postimmunization Measurement of *In Vivo* Specific Antibody Responses

Specific antibody responses can be evaluated by testing for spontaneous specific antibodies, such as isohemagglutinins, as well as antibodies to documented previous immunizations or

infections. The vaccine antibody response reflects an individual's ability to respond specifically to antigens contained in the vaccine. Thus, we must separately consider vaccines containing polysaccharide antigens, protein antigens and polysaccharide-conjugated-to-protein antigens, asg only the B lymphocyte response is involved in the first type and conjugated B and T cell responses are involved in the last two types. Therefore, the ability to respond to T-dependent and T-independent antigens must be investigated under suspicion of B cell deficiency. Another important topic to be considered is age, as distinct immune responses can be observed in infants, adults and elderly individuals. As a rule of thumb, adequate antibody titers to some of these vaccines in children up to 15 months old indicate a normal humoral immune response.

Protein Antigens (Tetanus Toxoid, Diphtheria Toxoid, and Measles/Mumps Serologies)

Tetanus and diphtheria toxoids are the main targets of the antibody response against protein antigens frequently used for PAD assessment. Their potent immunogenicity associated with the classic worldwide-accepted three-dose immunization program (with an acellular or cellular *Bordetella pertussis* component, rubeolla and tetanus toxoid) given to infants by six months of age helps explain this preference.

Tetanus and diphtheria titers above 0.1 and up to 0.2 IU/mL, respectively, are considered protective, and seroconversion rates approach 100% one month after the second or third dose (60, 61). Moreover, low levels after a vaccine booster in adult patients who have not been vaccinated for several years are expected, but children who have recently received routine immunization are expected to present a prominent response (62). Therefore, immunization records are crucial for interpreting vaccine responses.

Several other vaccine protein antigens are suitable for IEI diagnosis proposal and are shown to present well-established protective levels. Vaccination with inactivated live virus, such as hepatitis A, polio (inactive) and influenza, or recombinant antigens, such as hepatitis B, is safer and recommended for all immunocompromised patients. In contrast, vaccines with viable antigens, such as measles, mumps, polio (oral) and rubella, made with attenuated viruses require more attention with respect to adverse outcomes. Postvaccine antibody responses to measles and mumps are also commonly employed in the investigation of IEI, and levels >1.1 enzyme international units/mL (EIU/mL) are considered adequate, presenting seroconversion rates of 95–99% and 100% after the first and second doses, respectively (60). Protective levels of antibodies against protein antigens are well established and controlled by CAP. For this topic, we recommend the remarkable reviews by Bonilla et al. in 2015 and 2020 (16, 60).

Polysaccharide Antigens (*Streptococcus pneumoniae*)

Due to their low immunogenicity, most polysaccharide vaccines are conjugated to a protein or glycoprotein carrier to enhance antibody production. Specific T-independent antibody responses are mostly assessed after administration of a 23-valent capsular unconjugated polysaccharide vaccine, which includes the 23 most prevalent serotypes (PPV-23) (63). The response to

purified polysaccharide antigens is fully developed in 2-y/o children and the diagnosis of specific polysaccharide antibody deficiency (SPAD) must be considered in patients who receive PPV-23. If a previous immunization was performed with one of the conjugated vaccines, antibody titers for the other serotypes missing from the conjugated vaccine must be necessarily assessed, as protein conjugates induce antibodies indistinguishable from those induced by purified polysaccharides (62).

Serotype-specific IgG assessment by the well-standardized World Health Organization (WHO) ELISA is currently accepted as the 'gold standard' for the evaluation of antibody responses to pneumococcal serotypes (64), and this procedure recommends serum absorption with C-polysaccharide antigen and serotype 22 polysaccharide to remove nonprotective or cross-reactive antibodies. WHO ELISA has been shown to correlate closely with opsonophagocytosis assays (65), which is the only functional assay type available and is strongly correlated with vaccine efficacy. However, opsonophagocytosis assays are poorly used in clinical practice, as they have not been internationally standardized (66).

Antibody response against *S. pneumoniae* polysaccharide assessment is based on three main features: i) specific antibody levels increased over preimmunization levels; ii) the final concentration of antibodies after immunization; and iii) the percentage of serotypes against which an arbitrarily defined antibody titer was reached (67).

The protective serotype antibody level after protein-conjugated vaccines is not the same as that after PPV-23 vaccination. Protective levels are considered to be $\geq 0.35 \mu\text{g/mL}$ for each serotype after administration of protein-conjugated vaccines (68). After PPV-23 vaccination, children of 2 to 5 y/o are expected to develop at least a 2-fold increase in 50% of the serotypes tested, assuming that these levels are equal to or greater than $1.3 \mu\text{g/mL}$ (69). This cutoff level is considered protective against infection when assessed by ELISA, but the corresponding cutoff in multiplex immunoassay platforms has yet to be determined (66).

The multiplex addressable laser bead immunoassay (ALBIA) allows the simultaneous assessment of serum antibodies against the 23 pneumococcal polysaccharide serotypes present in PPV-23 (70–72). FDA-approved multiplex methods emerged in most reference laboratories as easier and faster attractive alternatives to ELISA that require significantly less sample volume, which is important in the pediatric population. Nonetheless, their correlations with WHO ELISA are variable, and significant result differences are reported by various laboratories (67, 73, 74).

Isohemagglutinins

Isohemagglutinins or allohemagglutinins have been proposed as alternatives to the determination of the pneumococcal polysaccharide vaccine response, as they are clinically relevant and inexpensive indicators of the ability to mount an antipolysaccharide response (75). Isohemagglutinins comprise naturally occurring IgM and IgG anti-polysaccharide antibodies that cross react with erythrocyte surface antigens A and B,

probably induced by contact with commensal gastrointestinal bacteria (76). Hence, these antibodies are usually undetected in newborns and patients with type AB blood. IgM and IgG isohemagglutinins can be ordered together or separately, and the results are semiquantitative and expressed in titers. Isohemagglutinin levels can be detected by 3–6 months of age, and 90% of adult titers are reached by 3 years of age, increasing to maximum levels between 5 and 10 years of age (77).

No cutoff values for isohemagglutinins are available. Most laboratories use a cutoff of 1/32 or 1/16. IEI specialists tend to use 1/8 for children below the age of 3 years and 1/16 for those above 3 years (78). Nevertheless, isohemagglutinins should not be used as a bona fide index of polysaccharide antibody response because different cutoff values (from 1/4 to 1/32) failed to discriminate individuals with SPAD from those with a normal Pn antibody response (78).

B Cell Immunophenotyping (Flow Cytometry)

Defects in B cell development, selection or function lead to humoral immunodeficiencies. With multiple surface marker staining, multiparametric flow cytometry can identify B cell subsets in peripheral blood, which, in turn, can be useful for PAD diagnosis. In addition, dynamic changes in the B cell compartment composition are observed during aging and may also be addressed. Studies have reported that, similar to other lymphocyte populations, total B cell counts increase by 2-fold immediately after birth, remain high until 2 years of age, and thereafter gradually decrease by approximately 6.5-fold until adulthood (79). On the other hand, age-related modifications of B cell maturation as well as clinically unvalidated immunophenotyping panels represent challenges for standardization and quality control. Moreover, accuracy differences in monoclonal antibodies and individual and population heterogeneity may also restrict reliable studies in this field.

Since the 1990s, a plethora of studies to standardize the total circulating B cell (CD19^+ or CD20^+) absolute count has been conducted within a wide age range in different populations (Table 3). Notably, an Italian national multicenter study defined reference ranges for normal values of CD19^+ B cells in a large cohort of 1,311 healthy adults (blood donors and volunteers chosen according to the Italian law for donor selection) (91). Despite no significant differences observed between hematology counters and cytometers, some methodological variables represented inevitable causes of variability, such as the quantity of sample, washing protocols, monoclonal antibodies and instrument brands used. Similar results were recently reported in healthy children aged 4 months to 7 years, as a Han Chinese initiative was accomplished (114).

In addition, as total circulating B cell assessment was rapidly popularized in immunology diagnostic labs around the world, quality control programs were needed to determine intra- and interlaboratory coefficients of variation, standardize procedures, define the best blood tubes and anticoagulants and, therefore, ensure comparable results, which was an issue predicted by studies in the late 1980s (121–124). The first robust initiative was conducted in 1990 with 3-year interlaboratory proficiency testing for lymphocyte subset phenotyping, consisting of part of the French Etalonorme national quality control program (125).

The authors concluded that calibration standards and instrument calibration procedures affect average cell counts; hence, the inclusion of lyophilized cells in each evaluation would offer a longitudinal approach for evaluating intra- and interlaboratory results. In 2000, the Belgian Scientific Institute of Public Health introduced a voluntary external quality assessment scheme for lymphocyte immunophenotyping, including CD19⁺ B cells, and demonstrated median intralaboratory coefficients of variation in cell percentages and absolute numbers of 3.2% and 16.5%, respectively (126). Although the topic was not discussed by the Belgian authors, one can argue that the higher absolute number intralaboratory variation observed may be caused by cell blood count variability, but this is an important bias to be solved. Later, a 10-year experience of expanded quality control study englobing all Benelux countries was published, and assay variability tended to decline with time (127). Currently, CAP

offers quality management programs by sending standard samples worldwide to voluntary participating labs and monitoring progress over time.

On the other hand, studies for the standardization and quality control of circulating B cell subsets are not as widespread as those for the total B cell count. Using CD27 as a surrogate marker of human memory B cells and CD38, immunoglobulin (Ig) M and IgD as differentiation markers, B cells have been divided into five different populations according to their differentiation stage in the lymphoid organs (128): naïve B cells (CD27⁻ IgD⁺); nonswitched memory B cells (CD27⁺ IgD⁺ IgM⁺); classical switched memory B cells (CD27⁺ IgD⁻ IgM⁻); transitional B cells (CD38^{high} IgM^{high}); and plasma cells (CD38^{high} IgM⁻). In addition, a CD21^{low} CD38^{low} B cell subset has been previously shown to be expanded in autoimmune diseases and immunodeficiencies (129, 130). Thus, due to its simplicity, this

TABLE 3 | Chronology of the main initiatives available in the medical literature for the standardization of total B cell circulating numbers, as rated according to the sample number, ethnicity and age range of recruited patients.

Authors	Year of publication	Sample (n)	Ethnicity	Age (y/o)	Ref.
Wiener et al.	1990	198	American	5-65	(80)
Reichert et al.	1991	271	Belgium, British, Swedish	18-70	(81)
Kotylo et al.	1993	130	American	0-17	(82)
Kontry et al.	1994	221	German	Newborns	(83)
Dhaliwal et al.	1995	152	Malay, Chinese and Indian	*	(84)
Roman et al.	1995	100	Romanian	Adults*	(85)
Kam et al.	1996	208	Chinese	18-71	(86)
Robinson et al.	1996	233	British	5-13	(87)
Comans-Bitter et al.	1997	429	Deutch	0-16	(88)
Huppert et al.	1998	513	British	64- >80*	(89)
Shahabuddin et al.	1998	132	Saudi Arabian	0-13; 18-44	(90)
Santagostino et al.	1999	1311	Italian	18-70	(91)
Al Qouzi et al.	2002	209	Saudi Arabian (male)	18-44	(92)
Kaaba et al.	2002	127	Kuwaiti Arab	18-59	(93)
Swaminathan et al.	2003	138	Indian	3-15	(94)
Shearer et al.	2003	807	American	0-18	(95)
Ikincioğullari et al.	2004	190	Turkish	0-18	(96)
Timová et al.	2004	495	Central and Eastern European	9-11	(97)
Chng et al.	2004	232	Singaporean (Chinese, Malay, Indian, Caucasian and Eurasian)	16-65	(98)
Bisset et al.	2004	70	Swiss	Adults*	(99)
Yaman et al.	2005	220	Turkish	18-80	(100)
Jentsch-Ullrich et al.	2005	100	German	19-85	(101)
Das Gupta A, Ochani Z	2006	185	Indian	18-49	(102)
Branch et al.	2006	112	Afro-Caribbean	Adults*	(103)
Al-Jabri et al.	2008	118	Omani (male)	18-51	(104)
Jiao et al.	2009	151	Chinese	19-83	(105)
Shoormasti et al.	2011	233	Iranian	20-45	(106)
Wong et al.	2013	273	Hong Kong Chinese	17-59	(107)
Al-Mawali et al.	2013	50	Omani	18-57	(108)
Kamallou et al.	2014	221	Iranian	20-40	(109)
Choi et al.	2014	294	Korean	21-80	(110)
Valiathan et al.	2014	150	American	12-18; 21-67	(111)
Valdiglesias et al.	2015	144	Spanish	65-95	(112)
Al-Thani et al.	2015	150	Qatari	18-55	(113)
Jia et al.	2015	1027	Han Chinese	0-7	(114)
Shahal-Zimra et al.	2016	326	Israeli	17-94	(115)
Qin et al.	2016	1068	Chinese	18-80	(116)
Azarsiz et al.	2017	90	Turkish	0-18	(117)
Kokuina et al.	2019	129	Cuban	18-80	(118)
EI Allam et al.	2020	83	Moroccan	0-18	(119)
Lerkvaleekul et al.	2020	182	Thai	0-15	(120)

*Exact data not available.

5-marker immunophenotyping panel (CD27, IgM, IgD, CD38 and CD21) has been commonly used to assess peripheral B cell maturation, and some standardization initiatives have already been conducted, although no quality control proposal is available to the best of our knowledge.

Based on this panel, Piatosa et al. (131) determined reference values for B cell subsets in healthy Polish children. Simultaneously, Morbach et al. (132) also established age-dependent reference values for distinct peripheral blood B cell populations in a cohort of individuals ranging from neonates to adults using the same immunophenotyping panel. Kverneland et al. (133) and Garcia-Prat et al. (134) determined reference values in adult Caucasian individuals older than 20 years and a pediatric Spanish population under 18 years, respectively. Although similar to that used by Piatosa et al. (131) and Morbach et al. (132), the immunophenotyping panel used by Kverneland et al. (133) and Garcia-Prat et al. (134) presented slight differences, including CD38^{dim} for class-switched and nonswitched memory B cells and CD24 expression for transitional cell and plasmablast assessment. Similarly, the EuroFlow PID group added two additional surface markers (CD5 and CD24) and conducted a comprehensive study addressing the distribution of normal B cell subsets in a wide age range: from cord blood to >80 y/o subjects (79). The EuroFlow staining strategy further subclassified memory B cells and plasma cells according to their membrane immunoglobulin isotype (IgG subclasses, IgA₁ and IgA₂). At first sight, these slight modifications seem innocuous; however, they are enough to impede comparison with previously cited studies.

Intracellular BTK Expression

X-linked agammaglobulinemia (XLA) is the most common form of inherited agammaglobulinemia, comprising 70% of all cases, and is caused by mutations in a pivotal protein for early pre-B cell receptor intracellular signaling: Bruton's tyrosine kinase (BTK). As XLA patients lack B cells, the deficient expression of mutated BTK can be assessed by flow cytometry using monocytes and platelets (135). Interestingly, this method is useful for the detection of not only XLA, but also BTK-deficient female carriers (136).

Although helpful for XLA diagnosis, monoclonal antibody standardization and optimal diagnostic cutoff values of intracellular BTK expression have yet to be determined, in turn requiring a simultaneous healthy control sample in every test. In addition, to our knowledge no reference range or interlaboratory quality assessment protocols have been determined.

Defective Cell Surface CVID-Related Protein Expression

CVID is the most common symptomatic PAD in adults, and diagnosis is mainly guided by clinical history, low immunoglobulin serum levels, defective vaccine responses and typical B cell immunophenotyping results. Approximately 30% of these cases may have an underlying genetic etiology, which, in turn, can be additionally confirmed by a flow cytometry-based assessment of the causative protein. At least 27 CVID-related monogenic conditions have been identified to date. Interestingly,

other cases carry variants of undetermined significance that can be validated by the same approach. Although specific CVID-causative mutated proteins represent only a fraction of all patients, some can be addressed by flow cytometry, such as TACI (137), BAFF-R (138), ICOS (139), CD19 (138), CD21 (140, 141), and ICOSL (142), among others. Nevertheless, these assays are not simple, as most require stimulation of the cells, and a few are seldom useful, for example, TACI (TNFRSF13b).

To date, we were not able to find any initiative for standardization or diagnostic accuracy assessment of such cell CVID-related surface protein expression. In addition, reference intervals and interlaboratory quality control programs for these methodologies have yet to be established. Hence, most labs recommend comparison with a simultaneously analyzed unrelated healthy control sample.

COMBINED IMMUNODEFICIENCIES OR IMMUNODEFICIENCIES AFFECTING CELLULAR AND HUMORAL IMMUNITY

SCID comprises a group of rare, monogenic disorders characterized by a blockade of the development of lymphoid stem cells into pre-T cells, with or without abnormal B and/or natural killer (NK) cell differentiation. Recently, several molecular defects causing SCID have been identified along with many other conditions causing incomplete T cell immunodeficiencies, which, in turn, are referred to as atypical SCID or, simply, combined immunodeficiencies (CID). This group of diseases presents early clinical manifestations with a spectral history of failure to thrive, unexplained diarrhea, interstitial pneumonitis, hepatosplenomegaly, oral candidiasis and other recurrent bacterial, viral, fungal or protozoal infections. The recent strategies comprising both early newborn screening and accurate diagnosis with lab tests detailed below allowed significant improvement in the proper specialized treatment and life expectancy of these patients (143, 144).

T Cell Receptor Excision Circles

Quantification of the copy number of T cell receptor excision circles (TREC) in peripheral blood, which is usually performed by quantitative real-time polymerase chain reaction (qRT-PCR), has been shown to be an effective tool for the early identification of severe T lymphocyte deficiencies. Quantitative analysis of TREC is frequently used to: i) estimate the thymopoiesis rate in newborn screening tests for SCID (145–147); ii) assess thymus involvement in autoimmune diseases (148, 149); and iii) evaluate T cell reconstitution during acquired immunodeficiency syndrome antiretroviral therapy and after bone marrow transplantation (150–154).

The TREC quantitative assay, initially proposed by Douek et al. (152) has been modified in different ways, which hampers result comparisons among different approaches. Newborn TREC quantification, which is performed using DNA extracted from dried blood spots, is a simple, low-cost methodology and

maintains sample stability, making it an ideal collection strategy (155). However, there are divergences among the different assays and units used to measure TREC, impeding interpretation and comparison among data. Values are often expressed as the absolute number of TREC molecules per μg of DNA within peripheral blood mononuclear cells or T lymphocytes or per 10^6 cells as an extrapolation of the recovery of 1 μg of DNA from approximately 150,000 cells (156). Another important issue is that there are no well-established age-specific reference intervals for SCID diagnosis, since most patients are infants and young children. Normally, an initial cutoff value for TREC quantification is used to determine whether a sample is within the normal range. Samples with TREC levels below the cutoff are usually sent for confirmatory tests (immunophenotyping of T cell subpopulations and genetic analyses). Each laboratory has established its own cutoff, as previous studies used a wide number of samples and advocated a screening sensitive cutoff of 25 copies/ μL , below which further clinical and laboratory investigation is required (157–159). Notably, the absence of a global reference range is not an issue, but a thorough standardization process in each lab is absolutely recommended. We suggest that a single cutoff may not be as representative as local reference intervals in healthy individuals of different age groups (145, 160). Therefore, it is important to establish cutoff values for assumed positive results based on assays using a sufficient number of samples (normal and diagnosed SCID cases) prior to test implementation as part of neonatal screening programs to avoid unnecessary patient recall.

T Cell Immunophenotyping

As HIV spread worldwide in late 1980s and CD4/CD8 T cell assessment proved useful in the management of AIDS patients, innumerable studies attempted to determine the reference range of total T cells and helper/cytotoxic subsets among different populations. **Table 4** summarizes the main initiatives to date to the best of our knowledge, albeit a comprehensive review of this topic would require an exclusive chapter. Simultaneously, external quality control and interlaboratory reproducibility assessment approaches were demanded during the 1990s, resulting in the organization of different national groups. One of the largest initiatives in the area was headed by the National Institute of Allergy and Infectious Diseases Division of AIDS (NIAID-DAIDS) (204), which, since 1999, has funded the Immunology Quality Assessment Program with the goal of assessing proficiency in basic lymphocyte subset immunophenotyping for each North American laboratory (205, 206). Nevertheless, other groups with similar purposes had previously succeeded not only in the United States (207) but also in Bulgaria (208), Italy (209) and the United Kingdom (210). Unsurprisingly, further initiatives developed afterward in Europe (126, 127), Africa (211–213), Asia (214, 215) and South America (216).

The steps in T cell maturation process are regulated by a complex transcriptional network, which mediates the homing, proliferation, survival, and differentiation of developing T cells (217–219). Therefore, unique combinations of surface markers can identify different T cell subsets with distinct functions (220).

In clinical practice, a CD45RA⁺/CD45RO⁺ imbalance toward memory T cells in a phenotypically suspected child may drive the diagnosis of combined immunodeficiencies. To the best of our knowledge, the first study aiming to standardize CD45RA⁺ T cells dates to 1992 in Spain (221). Other studies aiming to standardize the phenotyping of CD45RA⁺ naïve and CD45RO⁺ memory T cells have since been conducted in Kuwaiti Arabian (93), American (222), German (223), Italian (224), Dutch (225, 226), Brazilian (227) and Moroccan (228) healthy donors of different ages.

Although CD45RA, CD45RO, CD62L and C-C chemokine receptor 7 (CCR7) are the most common markers used for T cell maturation immunophenotyping indicated for CID diagnosis, the existence of several additional markers may result in challenging heterogeneity in laboratory reports from different services (218). Therefore, a consensus on the phenotypic definition of the various T cell subsets should be established, which will pave the way for robust standardization studies. Currently, different combinations of markers used to define such cells complicate comparability between studies and laboratories worldwide. **Table 5** shows standardization studies using mainly CD45RA/CD45RO/CCR7/CD62L-derived T cell subsets and, moreover, exemplifies the striking heterogeneity of immunophenotyping panels. Qin et al. (116) and Shearer et al. (95) determined the absolute number and percentage of T cell subsets using similar markers in the largest cohorts of adult and pediatric populations, respectively. Interestingly, the authors additionally determined the frequency of activation-primed (CD28⁺) and activated (HLA-DR⁺/CD38⁺) helper and cytotoxic T cells.

The multicentered EuroFlow and PERISCOPE (PERTussIS CORrelates of Protection Europe) consortia recently validated a 14-color immune monitoring flow cytometric tube capable of distinguishing more than 89 CD4⁺ T cell populations in peripheral blood, including several maturation and differentiation stages during aging, in 145 healthy donors (231). Unfortunately, despite comprehensive charts, no specific reference range was reported. A CAP quality assessment program is available for credited labs that voluntarily accept receiving regular heparinized whole blood samples to quantify CD45RA⁺ naïve, recent thymic emigrant (CD45RA⁺ CD31⁺), CD45RO⁺ memory and terminally differentiated effector memory (CD8⁺ CD45RA⁺ CCR7⁺) T cells. In addition to the low number of predefined T cell subsets, this strategy is also limited due to complications of cell viability in long-distance shipment and result comparability. Finally, to the best of our knowledge, no other quality assessment proposal is available regarding a broader T cell immunophenotyping panel.

Th17 Immunophenotyping

Th17 differentiation is mainly mediated by intracellular STAT3 activation. Therefore, STAT3 loss-of-function (LOF) or gain-of-function (GOF) mutations may equally impair circulating Th17 cell numbers in autosomal dominant hyper-IgE syndrome or autoimmune disease, multisystem, infantile-onset 1 (232). Despite the apparent usefulness of assessing Th17 cell numbers by flow cytometry for diagnostic purposes, a standard

TABLE 4 | Chronology of the main initiatives available in the medical literature for the standardization of circulating T cell numbers and CD4/CD8 subsets, as rated according to the sample number, ethnicity and age range of recruited patients.

Authors	Year of publication	Sample (n)	Cell population	Ethnicity	Age (y/o)	Ref.
Denny et al.	1992	208	CD3/CD4/CD8	American	1-5	(161)
Kotylo et al.	1993	130	CD3/CD4/CD8	American	0-17	(82)
Howard et al.	1996	215	CD3/CD4/CD8	American	18-67	(162)
Comans-Bitter et al.	1997	429	CD3/CD4/CD8	Deutch	0-16	(88)
Lisse et al.	1997	803	CD4/CD8	Bissau-Guinean	0-6	(163)
Shahabuddin et al.	1998	132	CD3/CD4/CD8	Saudi Arabian	0-13; 18-44	(90)
Tsegaye et al.	1999	485	CD3/CD4/CD8	Ethiopian	15-45	(164)
Al Qouzi et al.	2002	209	CD3/CD4/CD8	Saudi Arabian (male)	18-44	(92)
Swaminathan et al.	2003	138	CD3/CD4/CD8	Indian	3-15	(94)
Shearer et al.	2003	807	CD3/CD4/CD8	American	0-18	(95)
Uppal et al.	2003	94	CD4/CD8	Indian	18-74	(165)
Chng et al.	2004	232	CD3/CD4/CD8	Singaporean (Chinese, Malay, Indian, Caucasian and Eurasian)	16-65	(98)
Bisset et al.	2004	70	CD3/CD4/CD8	Swiss	Adults*	(99)
Lugada et al.	2004	3311	CD3/CD4/CD8	Ugandan	0-92	(166)
Bussmann et al.	2004	437	CD4/CD8	Botswanan	19-36	(167)
Jiang et al.	2004	614	CD4/CD8	Chinese	16-50	(168)
Amatya et al.	2004	200	CD3/CD4/CD8	Indian	18-55	(169)
Gomo et al.	2004	1113	CD4/CD8	Zimbabweans (pregnant)	Adults*	(170)
Yaman et al.	2005	220	CD3/CD4/CD8	Turkish	18-80	(100)
Jentsch-Ullrich et al.	2005	100	CD3/CD4/CD8	German	19-85	(101)
Aina et al.	2005	864	CD4	Nigerian	10-69	(171)
Ampofo et al.	2006	249	CD4/CD8	Ghanaian	18-83	(172)
Klose et al.	2007	186	CD4/CD8	Burkinabe	18-78	(173)
Al-Jabri et al.	2008	118	CD3/CD4/CD8	Omani	18-57	(104)
Das et al.	2008	252	CD3/CD4/CD8	Indian	Adults*	(174)
Ngowi et al.	2009	102	CD4/CD8	Tanzanian	Adults*	(175)
Murugavel et al.	2009	213	CD3/CD4/CD8	Indian	Adults*	(176)
Chama et al.	2009	541	CD4	Nigerian	Adults*	(177)
Oladepo et al.	2009	2570	CD4/CD8	Nigerian	18- >60*	(178)
Lawrie et al.	2009	678	CD4	South African	*	(179)
Buchanan et al.	2010	655	CD4/CD8	Tanzanian	0-18	(180)
Shoormasti et al.	2011	233	CD3/CD4/CD8	Iranian	20-45	(106)
Sagnia et al.	2011	352	CD3/CD4/CD8	Cameroonian	0-6	(181)
Thakar et al.	2011	1206	CD3/CD4	Indian	17-72	(182)
Pennap et al.	2011	444	CD4	Nigerian	15-44	(183)
Adoga et al.	2012	1123	CD3/CD4	Nigerian	0-50	(184)
Shakya et al.	2012	602	CD3/CD4/CD8	Nepalese	18-60	(185)
García-Dabrio et al.	2012	319	CD3/CD4/CD8	Spanish	4-88	(186)
Touil et al.	2012	242	CD3/CD4/CD8	Moroccan	19-49	(187)
Wong et al.	2013	273	CD3/CD4/CD8	Hong Kong Chinese	17-59	(107)
Al-Mawali et al.	2013	50	CD3/CD4/CD8	Omani	18-57	(108)
Moreno-Galván et al.	2013	400	CD3/CD4/CD8	Mexican	20-40	(188)
Torres et al.	2013	925	CD3/CD4/CD8	Brazilian	2-6; 19-56	(189)
Kamallou et al.	2014	221	CD3/CD4/CD8	Iranian	20-40	(109)
Valiathan et al.	2014	150	CD3/CD4/CD8	American	12-18; 21-67	(111)
Atanasova et al.	2014	72	CD3/CD4/CD8	Bulgarian	Newborns	(190)
Tembe et al.	2014	257	CD3/CD4/CD8	Mozambican	18-24	(191)
Jia et al.	2015	1027	CD3/CD4/CD8	Han Chinese	0-7	(114)
Al-Thani et al.	2015	150	CD3/CD4/CD8	Qatari	18-55	(113)
Prasetyo et al.	2015	241	CD4	Javanese	18-65	(192)
Shahal-Zimra et al.	2016	326	CD3/CD4/CD8	Israeli	17-94	(115)
Qin et al.	2016	1068	CD3/CD4/CD8	Chinese	18-80	(116)
Zhang et al.	2016	268	CD3/CD4/CD8	Chinese	21-60	(193)
Afolabi et al.	2017	1205	CD4	Nigerian	0-65	(194)
Mulu et al.	2017	481	CD4	Ethiopian	18-65	(195)
Yeshanew et al.	2017	400	CD3/CD4/CD8	Ethiopian (pregnant)	18-40	(196)
Genetu et al.	2017	200	CD4	Ethiopian (pregnant)	18-42	(197)
Enawgaw et al.	2018	967	CD4	Ethiopian	18-61	(198)
Karn et al.	2018	207	CD3/CD4	Nepalese	0-14	(199)
Kokuina et al.	2019	129	CD3/CD4/CD8	Cuban	18-80	(118)
Louati et al.	2019	143	CD3/CD4/CD8	Tunisian	18- >45*	(200)
Mishra et al.	2020	400	CD3/CD4	Nepalese	15-60	(201)
Niu et al.	2020	150	CD4	Han Chinese	20-70	(202)
Scheffer-Mendoza et al.	2020	50	CD3/CD4/CD8	Mexican	Newborns	(203)

*Exact data not available.

TABLE 5 | Chronology of the main initiatives available in the medical literature for the standardization of circulating naïve and memory T cell subsets, as rated according to the sample number, ethnicity and age range of recruited patients.

Authors	Year of publication	Sample (n)	T cell subset markers	Ethnicity	Age (y/o)	Ref.
Shearer et al.	2003	807	CD45RA CD45RO CD62L HLA-DR CD38	American	0-18	(95)
Bisset et al.	2004	70	CD45RA CD45RO CD62L HLA-DR CD38	Swiss	Adults*	(99)
Jiao et al.	2009	151	CD45RA CD45RO CD62L HLA-DR CD28 CD38	Chinese	19-83	(105)
Sagnia et al.	2011	352	CD45RA CD45RO CD62L HLA-DR CD38	Cameroonian	0-6	(181)
Moraes-Pinto et al.	2014	463	CD45RA CCR7 CD38 CD27	Brazilian	0-48	(229)
Valiathan et al.	2014	150	CD45RA CD45RO CD62L HLA-DR CD28 CD38	American	12-18; 21-67	(111)
Bretschneider et al.	2014	66	CD45RA CCR7 CD27 CD57	German	0-72	(230)
Qin et al.	2016	1068	CD45RA CD45RO CD62L HLA-DR CD28 CD38	Chinese	18-80	(116)
Garcia-Prat et al.	2019	159	CD45RA CD45RO CCR7	Spanish	0-18	(134)

The immunophenotyping panel used by each paper is also depicted, which consisted of different combinations of the staining markers CD45RA, CD45RO, CCR7, CD62L and HLA-DR. *Exact data not available.

immunophenotyping panel has yet to be defined. Moreover, a validated reference range for circulating Th17 cell numbers is usually unavailable, which makes running a simultaneous healthy control sample mandatory for result comparison. Botafogo et al. (231) recently analyzed 113 samples from healthy controls aged 0-89 years to establish reference values for Th17 cells defined as CD183⁻/CD194⁺/CD196⁺/CCR10⁻. According to the authors, these cell surface markers were proven accurate in identifying IL17A-producing cells. Similarly, Niu et al. (202) established distributions and reference ranges for stimulated CD4⁺ IL17-producing cells in 150 healthy Chinese healthy volunteers aged 20-70 years. However, we were not able to find more data regarding Th17 cell reference ranges in other populations. In addition, no

quality assessment program for Th17 immunophenotyping is available.

Intracellular Wiskott-Aldrich Protein Expression

Flow cytometry-based assessment of intracellular WAS protein (WASP) is useful for screening patients suspected to have WAS or X-linked thrombocytopenia and neutropenia (233) and for following up chimerism after hematopoietic stem cell transplantation or somatic reversion mosaicism (234). Despite its use in immunology clinics worldwide, methodology standardization, diagnostic accuracy and optimal diagnostic cutoff values for flow cytometric WASP measurement are still

lacking. Similarly, reference intervals and interlaboratory quality assessment programs for intracellular WASP expression have not been determined. Therefore, a simultaneous healthy control sample run is recommended to validate results (Figure 1). Recently, Rawat et al. (235) suggested a stain index ratio using the median fluorescence intensities of patients and controls and found that values lower than 0.65 for gated lymphocytes are suggestive of WAS. Regardless, a broader validation of other centers is still needed.

Defective Cell Surface or Intracellular SCID-Related Protein Expression

SCID diagnosis is mainly guided by clinical history, newborn TREC screening, typical T cell immunophenotyping results and potentially impaired lymphoproliferation in response to mitogens. Once a diagnosis is made, gene sequencing analyses may determine the underlying etiology, which can also be confirmed by a flow cytometry-based assessment of the defective protein. On the other hand, although approximately 2030% of those cases remain without any identifiable pathogenic mutation (236, 237), some carry variants of undetermined significance, which, in turn, can be validated by appropriate assays.

More than 50 SCID-causative molecular targets have been identified to date. Similar to specific PAD-causative proteins, some molecules are qualified to be addressed by a flow cytometric CID and SCID-driven diagnostic approaches, namely, CD132 (IL-2R γ) (238), CD127 (IL-7R α) (239), major histocompatibility complex I (240) and II (241), CD45 (242), CD3 chains (239), DOCK8 (243), and IKAROS (244).

Despite our lack of intention to exhaust this topic and the natural difficulty of validating a methodology for an uncommon condition with even rarer subtypes, we are not aware of available protocols for test standardization and quality assessment programs thus far. Moreover, reference intervals for defective cell surface and

intracellular SCID-related protein expression are still lacking. Therefore, for the analytes discussed above, a simultaneous healthy control sample run is pivotal for comparison.

Adenosine Deaminase and Purine Nucleoside Phosphorylase Activity

Adenosine deaminase 1 (ADA-1) deficiency is an autosomal recessive disorder resulting in a heterogeneous form of combined immunodeficiency. Specific diagnosis of ADA-1 deficiency in immunodeficient patients can be achieved by enzyme activity or metabolite quantification assays of several easily available cell types, usually erythrocytes. Affected individuals have less than 1% normal ADA-1 catalytic activity in red cell hemolysates. Kinetic ADA activity assays have been extensively reproduced since their initial description (245–250), allowing companies to develop fluorometric and spectrophotometric assays. Despite commercially available standardized tests, diagnostic accuracies and reference ranges remain unestablished. This may partially explain several reports of ADA-1-deficient patients without immunodeficiency (251, 252). Of note, to the best of our knowledge, only two small uncontrolled studies have systematically determined ADA-1 erythrocyte activity in healthy controls (253, 254). No specific quality assessment program for ADA-1 erythrocyte activity has been proposed to date.

Similar to ADA-1, purine nucleoside phosphorylase (PNP) participates in the purine salvage pathway. PNP deficiency can result in a rare CID with associated syndromic features. Low PNP activity in erythrocyte lysates can be assessed by liquid chromatography-tandem mass spectrometry and enzymatic colorimetric assay (255, 256). However, to our knowledge, no study determining reference values in a healthy control population or establishing a quality assessment has been found.

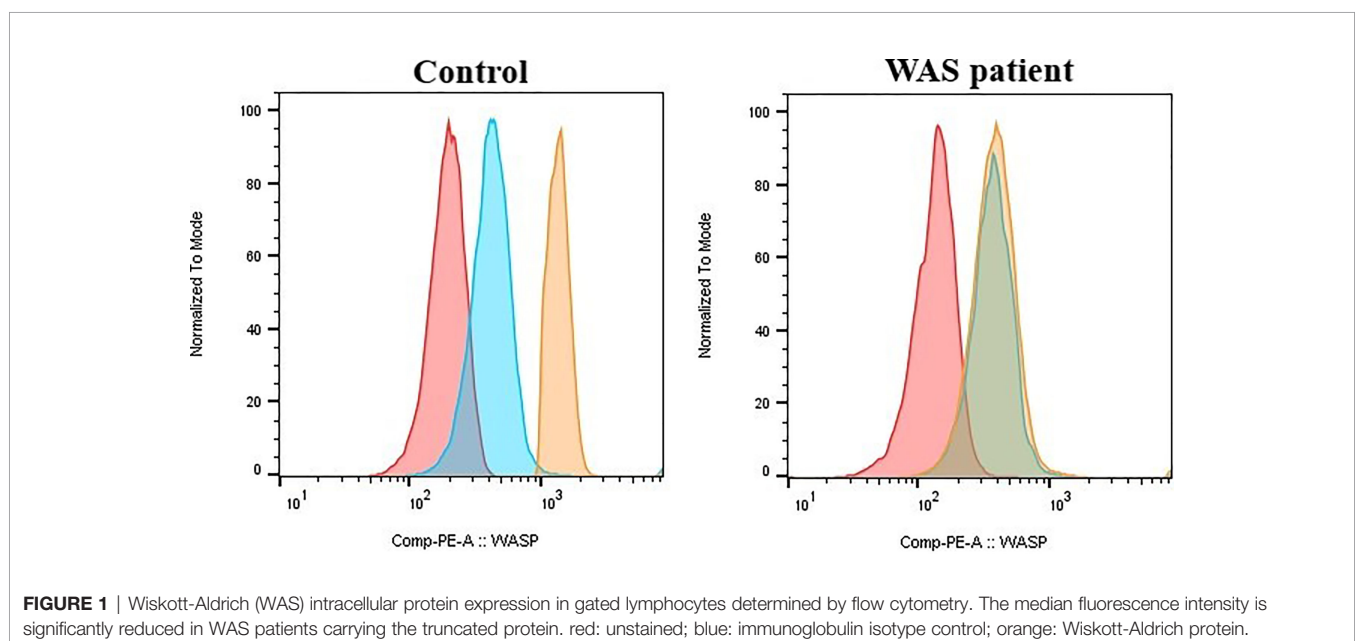


FIGURE 1 | Wiskott-Aldrich (WAS) intracellular protein expression in gated lymphocytes determined by flow cytometry. The median fluorescence intensity is significantly reduced in WAS patients carrying the truncated protein. red: unstained; blue: immunoglobulin isotype control; orange: Wiskott-Aldrich protein.

DISEASES OF IMMUNE DYSREGULATION

Approximately 30% of all monogenic IEI described thus far have a clinical phenotype predominantly resulting from a maladaptive change in molecular control leading to immune regulation breakdown, such as autoimmunity, autoinflammation, lymphoproliferation, malignancy and severe atopy, rather than infections (1). This group of disorders is rapidly growing and has been recently termed primary immune regulatory disorders (PIRD) (1, 257). Despite limited importance, routine nonfunctional immunology labs can be helpful under specific situations, as follows.

Hemophagocytic Lymphohistiocytosis

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening hyperinflammatory disease mainly in children younger than 1 year manifested by high persistent fever, pancytopenia, hepatosplenomegaly, and elevated aminotransferase and ferritin levels (258). The cytotoxic activity of CD8⁺ T cells and natural killer (NK) cells is impaired in primary HLH, impeding the elimination of virus-infected cells and instead causing continuous secretion of inflammatory cytokines, especially soluble CD25.

Soluble CD25

CD25 is strongly expressed after T cell activation. Upon activation, a 40-45-kD truncated protein is cleaved off of the 55-kD IL-2R α membrane protein and shed into circulation as the soluble IL-2 receptor (sIL-2R or sCD25) (259). Thus, sCD25 is considered a surrogate marker of T cell activation, and elevated serum levels have been described in various diseases, including hematological malignancies (e.g., HTLV-1-associated T cell leukemia, hairy cell leukemia, acute lymphoblastic leukemia, and non-Hodgkin lymphoma), infections (e.g., human immunodeficiency virus, viral hepatitis, and Epstein-Barr virus), autoimmune conditions (e.g., rheumatoid arthritis, sarcoidosis, systemic lupus erythematosus, systemic juvenile idiopathic arthritis, Kawasaki's disease, and autoimmune lymphoproliferative syndrome/ALPS), allograft rejection and graft-vs.-host disease after allogeneic hematopoietic stem cell transplantation (260, 261). In addition, sCD25 is also released from dendritic cells, activated B cells, monocytes, and malignant cells (260). sCD25 levels have been incorporated as one of the eight laboratory and clinical criteria for HLH diagnosis, of which five must be met for diagnosis.

The currently available methods for sCD25 assessment are ELISA, whose results are expressed as pg/mL, and chemiluminescent immunoassay (ChLIA), with results expressed as U/mL. Although these assays present good correlation, the differing units may cause confusion. The cutoff has been defined as 2400 U/mL for pediatric patients, which may correspond to approximately 20,000 pg/mL in ELISA. A similar cutoff has been defined for adults (262), although it has been described that ELISA-determined sCD25 levels are higher in children (age 1-14 years) and elderly individuals (age 67-99 years) than in adults (age 22-67) (263, 264). The normal range in adults was set as 241-846 U/mL (265).

Damoiseaux et al. (259) reported a ChLIA sCD25 cutoff of 600 U/mL, which is equivalent to an ELISA cutoff between 4200 and 4800 pg/mL. Most clinical laboratories have set ELISA cutoffs between 2500 and 3500 pg/mL, although different strategies for sCD25 serum level cutoff standardization are adopted, generally based on the mean plus two standard deviations. Repeated sCD25 serum level assessment is also helpful for treatment monitoring and prognostic risk scoring in several conditions (261).

Intracellular Protein Expression – PRF1, SAP/SH2DIA, XIAP

(266) Some forms of primary HLH present defects that can be assessed by flow cytometry (267). One of these targets is perforin, which is easily quantified by intracellular staining flow cytometry; moreover, defects in granule transport can be screened by CD107a (LAMP1) exocytosis evaluated by flow cytometry (268, 269), as well as X-linked lymphoproliferative (XLP) analysis (270, 271). Once again, despite our lack of intention to exhaust this topic and the natural difficulty of validating a methodology for an uncommon condition, to the best of our knowledge, no protocols for test standardization and quality assessment programs are available thus far.

ALPS

Autoimmune lymphoproliferative syndrome (ALPS) is a group of human disorders caused by genetic defects disrupting lymphocyte apoptosis (272). Currently, this expanding group of disorders includes prototypical autoimmune lymphoproliferative syndrome (ALPS, OMIM #601859), which is caused by defects in the FAS pathway of apoptosis (*FAS*, *FASLG*, and *CASP10*), and RAS-associated autoimmune leukoproliferative disorder (RALD), which is caused by somatic mutations in *NRAS* or *KRAS*. Most patients harbor pathogenic variants in the *FAS* gene inherited in an autosomal dominant fashion (272). Typical clinical findings include benign, chronic lymphadenopathy and splenomegaly; autoimmune cytopenias; and a high risk for lymphoma development (273). The classical laboratory hallmark of ALPS is the presence of circulating mature α/β receptor-carrying T cells that do not express CD4 or CD8 (double-negative T cells), which is a finding required for diagnosis (274). Other common laboratory manifestations include hypergammaglobulinemia, the presence of autoantibodies directed to blood cell elements, high levels of vitamin B12 and increase in soluble cytokines such as IL-10, IL-18 and soluble Fas ligand.

Double-Negative TCR α/β Circulating T Cells (DNT)

As a hallmark and required diagnostic finding in ALPS, the measurement of circulating double-negative T cells (DNT) is performed by flow cytometry (275, 276). This assay is easily conducted with a four-color instrument, and standardization requires running a panel of normal individuals to define the normal range in a particular laboratory, as is common practice for other flow cytometry assays. Gating was performed using T cell receptor (TCR) α/β , CD3, CD4 and CD8 staining. Values of CD3⁺ TCR $\alpha\beta$ ⁺ CD4⁻ CD8⁻ DNT cells above \geq 1.5% of total lymphocytes or 2.5% of CD3⁺ lymphocytes in the setting of

normal or elevated lymphocyte counts are considered abnormal, but these values may vary slightly among particular laboratories (274). Once established, the assay can undergo external quality assessment by interlaboratory exchanges, as many labs around the country perform the assay. There are no commercially available CAP controls for this measurement.

Soluble Mediators: IL-10, IL-18, Soluble FASL and Vitamin B12

The elevation of soluble cytokines and vitamin B12 was noted in ALPS patients early, particularly in those with *FAS* mutations (277, 278). These levels were later systematically measured in a large cohort of patients and controls and noted to have high positive and negative predictive values for the presence of *FAS* mutations (278). In particular, the combination of high DNT cells with elevated soluble FASL was shown to be a very potent predictor of *FAS* LOF mutations (278). Measurements of IL-10, IL-18 and sFASL can easily be performed by ELISA or ALBIA or similar protein immunoassays. A panel of controls should be run to define the range of normal values, and external quality assessment can be performed by interlaboratory sample exchanges.

IPEX

IPEX syndrome is a rare monogenic primary immunodeficiency caused by *FOXP3* LOF mutations, which encodes a pivotal transcription factor required for the development of regulatory T cells. Treg cell absence or dysfunction are the main pathogenic events associated with early onset multiorgan autoimmunity in IPEX. We will discuss the main findings on standardization and quality assessment for circulating Treg cell numbers.

T Regulatory Cell Number

Several immunophenotyping panels have been suggested to discriminate circulating Treg cells. Despite controversies regarding the most appropriate panel, the literature has lately converged to a 4-marker panel: CD4⁺/CD25⁺/CD127^{low}/Foxp3⁺. A consensus on the immunophenotyping definition based on 40 European and American experts was recently proposed and included a robust gating strategy for the context-dependent analysis of Tregs by flow cytometry (279). Later, a French initiative provided a perspective on methodological standardization and analysis using human Treg data obtained from healthy donors, transplanted patients, and, furthermore, parallel standard murine strains (C57BL/6 and BALB/c) (280). Recent studies also standardized the flow cytometry procedure for monitoring Treg cells stained with the CD4⁺/CD25⁺/CD127^{low}/Foxp3⁺ panel associated with other markers (281, 282).

Regarding normal range standardization, Kim et al. (283) established reference intervals for CD4⁺/CD25^{high}/Foxp3⁺ Treg cells in umbilical cord blood from 120 healthy neonates, highlighting that Treg cell numbers are higher in newborns, particularly in premature infants (284). Moreover, Niu et al. (202) recently determined the reference ranges of circulating Treg cells in 150 gender-balanced healthy adults of the Han Chinese population aged 20-70 years. Nevertheless, to the best of our knowledge, no additional data are available regarding Treg cell reference ranges in other populations or quality assessment

programs. Therefore, a concurrent healthy control sample run is mandatory to determine whether Foxp3 expression is comparable.

DEFECTS IN PHAGOCYTES, AND INTRINSIC AND INNATE IMMUNITY

The main feature of the innate immune system relies upon a limited germline repertoire of alarmins and receptors for common biochemical signature detection of danger and invading pathogens. Innate immunity receptor-induced intracellular signaling and cell activation are not restricted to the immune system, but also include nonhematopoietic cells. Therefore, defects in intrinsic and innate immunity encompass a heterogeneous group of disorders with systemic susceptibility to specific categories of infectious agents, such as mycobacteria, invasive pyogenic bacteria, viruses, parasites, and fungi. On the other hand, congenital impairment of phagocytes, as the main innate immunity effector cells, is associated with a similar clinical phenotype. Some monogenic conditions encoding truncated proteins classified within these two groups of diseases may be identified by flow cytometry-based assays.

MSMD

Mendelian susceptibility to mycobacterial diseases (MSMD) is a group of approximately 30 different diseases associated with mutations in 15 genes, presenting inherited susceptibility to BCG and environmental atypical mycobacteriosis (285). The first diseases described in this group were defects in the expression of interferon-gamma alpha and beta chains (IFNGR1 and IFNGR2), followed by deficiencies in interleukin-12/23 beta 1 chain (IL12RB1) and STAT-1. Some of these diseases can be evaluated by flow cytometric expression of the molecules *ex vivo* or after stimulation.

Cell Surface and Cytoplasmic Protein Expression: IFN γ -R1, IFN γ -R2, IL12-RB1 and STAT-1

The first described diseases among MSMD, namely, IFN γ receptor and IL-12/23 receptor beta chain 1 deficiencies, can be easily evaluated by flow cytometry (286). IFN γ receptor alpha chain (IFN γ -R1 or CD119) can be evaluated by the expression of CD119 on monocytes, and T lymphocytes. Partial dominant negative IFN γ -R1 deficiency is usually characterized by overexpression of the receptor due to the lack of an intracellular domain region associated with impaired recycling of the molecule (287). Phosphorylated STAT-1 expression can be assessed by intracellular flow cytometry (288). Deficiency in the beta-1 chain of IL12/23 receptor (IL12RB1 or CD212) is the most common form of MSMD (289) and can be evaluated by flow cytometry after activation of T cells, somehow increasing the complexity of the evaluation and the possibility of standardization. The same approach is important to the evaluation of IL12RB2 protein expression, but this disease is very rare and has been described only recently (285). As for most of the extremely rare conditions described above, no protocols for test standardization and quality assessment programs are available to date (290).

LAD

Leukocyte adhesion deficiency (LAD) syndromes are very rare autosomal recessive diseases characterized by leukocytosis associated or not with other clinical and laboratory features (291). There are three forms of LAD, namely, LAD1, 2 and 3, with different genetics and pathophysiology (292, 293). LAD1 is associated with mutations in *ITGB2*, which is the gene for the beta chain of beta-2 integrins, also known as CD18, and mediates cell-cell and cell-extracellular matrix adhesion (294, 295). Therefore, LAD1 patients present leukocytosis with

neutrophilia associated with recurrent bacterial infections and impaired pus formation and wound healing (296). LAD2 is associated with the mutation of *SLC35C1*, which is a gene encoding a GDP-fucose transmembrane transporter (FucT1). It is characterized by leukocyte adhesion defects associated with severe mental and growth retardation. LAD2 is also known as a congenital disorder of glycosylation type IIc (297). LAD3 is caused by mutations in the *FERMT3* (or *KINDLIN3*) gene, presenting a leukocyte adhesion defect with delayed umbilical cord detaching, omphalitis, severe bacterial infections, and

TABLE 6 | Quality and standardization control stratification of inborn errors of immunity (IEI) assessment nonfunctional immunoassays.

IEI categories	Nonfunctional immunoassay	Method	Method standardization	Quality control program	Reference range (including early age groups)	
Predominantly antibody deficiencies	IgG, IgM and IgA	Nephelometry or turbidimetry	Standardized	Established	Standardized	
	IgD	ELISA	Standardized	Established	Nonstandardized	
	IgE	ELISA or fluorimetry	Standardized	Established	Few initiatives	
	IgG subclasses	Nephelometry or turbidimetry	Standardized	Established	Standardized	
	Salivary IgA	ELISA	Few initiatives	Unestablished	Few initiatives	
	Vaccine response against tetanus toxoid	ELISA	Standardized	Unestablished	Few initiatives	
	Vaccine response against diphtheria toxoid	ELISA	Standardized	Unestablished	Few initiatives	
	Vaccine response against measles	ELISA	Standardized	Unestablished	Standardized	
	Vaccine response against mumps	ELISA	Standardized	Unestablished	Standardized	
	<i>In vivo</i> vaccine response against <i>Streptococcus pneumoniae</i>	ELISA	Standardized	Established	Standardized	
		Multiplex	Standardized	Unestablished	Nonstandardized	
	Isohemagglutinins	Hemagglutination	Standardized	Unestablished	Few initiatives	
	B cells (CD19 ⁺ or CD20 ⁺)	Flow cytometry	Standardized	Established	Standardized	
	B cell immune phenotyping	Flow cytometry	Few initiatives	Unestablished	Few initiatives	
	Intracellular BTK expression	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized	
Defective cell surface CVID-related protein expression	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized		
Combined immunodeficiencies	TREC	qRT-PCR	Standardized	Unestablished	Few initiatives	
	CD4 ⁺ /CD8 ⁺ T cells	Flow cytometry	Standardized	Established	Standardized	
	T cell immune phenotyping	Flow cytometry	Few initiatives	Unestablished	Few initiatives	
	Th17 immunophenotyping	Flow cytometry	Nonstandardized	Unestablished	Few initiatives	
	Intracellular Wiskott-Aldrich protein expression	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized	
	Defective cell surface or intracellular SCID-related protein expression	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized	
	ADA-1 erythrocyte activity	Fluorometry, spectrophotometry	Nonstandardized	Unestablished	Nonstandardized	
	PNP erythrocyte activity	LCTMS, ECA	Nonstandardized	Unestablished	Nonstandardized	
	Soluble CD25	ELISA, chemoluminescence	Standardized	Unestablished	Few initiatives	
	Defective intracellular HLH-related protein expression (PRF1, SAP/SH2DIA, XIAP)	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized	
Diseases of immune dysregulation	Double negative TCR α/β circulating T cells (DNT)	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized	
	IL-10	ELISA, ALBIA	Standardized	Unestablished	Few initiatives	
	IL-18	ELISA, ALBIA	Standardized	Unestablished	Few initiatives	
	Soluble FASL	ELISA, ALBIA	Standardized	Unestablished	Few initiatives	
	B12 vitamin	ELISA	Standardized	Established	Standardized	
	Defects in phagocytes, intrinsic and innate immunity	Cell surface protein expression: IFN γ -R1 and IFN γ -R2	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized
		Cell surface protein expression: CD18, CD11a/CD11b/CD11c, CD15	Flow cytometry	Nonstandardized	Unestablished	Nonstandardized

The main methodologies platforms used for each test are also presented. Standardization and quality control stratification for each test are rated as: "standardized/established", in the case of robust, clear data available; "few initiatives", in the case of only reports or low-numbered uncontrolled case series available; or "nonstandardized/unestablished", in the case of no trustworthy data available.

ADA-1, adenosine deaminase-1; ALBIA, addressable laser bead immunoassay; ECA, enzymatic colorimetric assay; ELISA, enzyme-linked immunosorbent assay; HLH, hemophagocytic lymphohistiocytosis; LCTMS, liquid chromatography-tandem mass spectrometry; PNP, purine nucleoside phosphorylase; PRF1, perforin 1; SAP/SH2DIA, SLAM-associated protein/SH2 domain-containing protein 1A; TREC, T cell receptor excision circles; XIAP, X-linked inhibitor of apoptosis.

delayed wound healing and associated with bleeding tendency with normal platelet numbers (298).

Cell Surface Protein Expression: CD18, CD11a/CD11b/CD11c, CD15

Screening of leukocyte adhesion defects can be performed by simple flow cytometry techniques (299). CD18 is present in all lineages of nucleated hematopoietic cells, and its absence is typical of LAD1. LAD2 can be diagnosed by the presence of the Bombay phenotype due to the absence of the H antigen in red blood cells, therefore applying to blood types A, B, AB and O. Another characteristic is the absence of CD15s (sialyl-Lewis Ag) in flow cytometry. Finally, LAD3 can be screened by the absence of beta-1 and beta-2 integrins in flow cytometry of platelets and phagocytes. However, to our knowledge, none of these methodologies are standardized or have any quality assessment programs available.

CONCLUSIONS

Standardization and quality assessment programs are pivotal for immunology diagnostic tests, especially those targeting “lab-dependent” identification of disorders routinely assessed by IEI-specialized clinical immunologists. Nonfunctional tests are generally a good alternative for relatively low-cost, quick and definitive

diagnoses. Despite the rich literature describing anecdotal cases or series reports for each specific assay shown in this manuscript, unfortunately, most lack robust methodological and populational standardization. **Table 6** summarizes all nonfunctional immunoassays herein listed and stratifies them according to the presence or absence of standardization and quality assessment initiatives. The popularization and reliability of nonfunctional immunoassays should be enhanced by multicenter collaborative studies addressing methodological standardization and the establishment of reference ranges and quality assessment programs.

AUTHOR CONTRIBUTIONS

SP, PP, DM-V, JO, LA, and MC-S contributed to conception and design of the study. SP organized the contents and wrote the first draft of the manuscript. All authors wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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GLOSSARY

ADA-1	Adenosine deaminase 1
ALBIA	addressable laser bead immunoassay
ALPS	autoimmune lymphoproliferative syndrome
ATM	ataxia-telangiectasia
BTK	Bruton's tyrosine kinase
CAP	College of American Pathologists
CCR7	C-C chemokine receptor 7
CI	confidence intervals
CID	combined immunodeficiencies
CLIA	Clinical Laboratory Improvement Amendments
ChLIA	chemiluminescent immuno-assay
CVID	common variable immunodeficiency
DNT	double negative TCR alpha/beta circulating T cells
DOCK8	dedicator of cytokinesis 8
EIU/mL	enzyme international units/mL
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic <i>E. coli</i>
FDA	Food and drug administration
GOF	gain-of-function
HIDS	hyper-IgD syndrome
HLH	hemophagocytic lymphohistiocytosis
IEI	inborn errors of immunity
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked
IUIS	International Union of Immunological Societies
LAD	leukocyte adhesion deficiency
LOF	loss-of-function
MKD	mevalonate kinase deficiency
MSMD	Mendelian susceptibility to mycobacterial diseases
NIAID-DAIDS	National Institute of Allergy and Infectious Diseases Division of AIDS
NK	natural killer
PAD	predominantly antibody deficiency
PERISCOPE	PERTussIS COrrelates of Protection Europe
PID	primary immunodeficiency diseases
PIRD	primary immune regulatory disorders
PNP	purine nucleoside phosphorylase
PnPS	pneumococcal polysaccharide
PPV-23	pneumococcal prevalent 23 serotypes
QAS	quality assessment
qRT-PCR	quantitative real-time polymerase chain reaction
RALD	RAS-associated autoimmune leukoproliferative disorder
SCID	severe combined immunodeficiency
SD	standard deviation
slgA	secretory IgA
SIgAD	selective IgA deficiency
SPAD	specific polysaccharide antibody deficiency
TCR	T cell receptor
TREC	T cell receptor excision circles
Treg	regulatory T cells
TYK2	tyrosine kinase 2
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WHO	World Health Organization
XLA	X-linked agammaglobulinemia
XLP	X-linked lymphoproliferative syndrome