

Molecular- and Flow Cytometry-based Diagnosis of Primary Immunodeficiency Disorders

Joao B. Oliveira · Thomas A. Fleisher

© US Government 2010

Abstract Primary immunodeficiencies are an expanding group of genetic disorders resulting in recurrent and/or severe infections, autoimmunity, or autoinflammation. The laboratory plays a critical role in the diagnosis of these conditions given their frequently overlapping signs and symptoms. We discuss here advances in flow cytometry and molecular techniques applied to the study of primary immunodeficiencies.

Keywords Flow cytometry · Genetics · Primary immunodeficiency · Diagnosis · Molecular

Introduction

Flow cytometry provides a clinical tool for evaluating the immune system that can detect the absence of a specific cell population or subpopulation, screen for altered expression of a specific extracellular or intracellular protein, assess for biological changes associated with specific immune defects, and evaluate certain functional immune characteristics. Applied in the setting of possible immune deficiency, this technology can in some cases clarify a diagnosis and in other settings help direct additional testing to establish a diagnosis. More recently, molecular methods have emerged as important diagnostic tools in the evaluation of patients

with possible immune deficiency. In this review, very basic principles of flow cytometry are outlined together with a discussion of disease-specific applications. This is followed by a brief review of current methods used for mutation analysis as well as a discussion of T-cell receptor excision circle (TREC) quantitation and T-cell repertoire/diversity evaluation, all in the setting of applications for evaluating possible immune deficiency.

Flow Cytometry

The design specifics of a flow cytometer are beyond the scope of this article, but briefly, the instrument has four major elements: optics, fluidics, electronics, and a computer (with specific software) [1]. The optical system uses monochromatic light sources (typically lasers) that provide the excitation energy. The optical bench collects light derived directly from each cell as it passes through the laser beam(s). Each cell emits nonfluorescent (forward and side scatter) as well as fluorescent signals if one or more fluorochrome conjugated monoclonal antibodies are bound to the cell. The two nonfluorescent parameters collected provide an index of cell size (forward light scatter) and a measure of cell granularity/regularity (side-angle light scatter). The combination of these two parameters allows for a “three-part leukocyte differential,” which in nonmalignant settings easily distinguishes among lymphocytes, monocytes, and granulocytes in a whole blood sample following red blood cell lysis [2].

The fluorescent signals result from cell surface or intracellular binding of specific monoclonal antibodies conjugated directly to fluorochromes that, following excitation by a specific wavelength, emit light of lower energy

J. B. Oliveira · T. A. Fleisher (✉)
Department of Laboratory Medicine, Clinical Center,
National Institutes of Health,
NIH Building 10, Room 2C306, 10 Center Drive, MSC 1508,
Bethesda, MD 20892, USA
e-mail: tfleishe@mail.nih.gov

J. B. Oliveira
e-mail: oliveirajb@cc.nih.gov

(longer wavelength). The availability of multiple fluorochromes excited by the same wavelength with emissions of different wavelengths facilitates the use of multiple reagents simultaneously for a multicolor study with a single light source. A second laser is typical in most clinical instruments, facilitating additional “colors,” and three laser instruments have become more common for clinical studies, readily allowing eight “color” studies. These polychromatic studies often include reagents that combine evaluation of cell surface and intracellular targets. Recent descriptions of new fluorochromes and modified instrumentation have pushed reported multicolor applications for clinical studies to 10 “colors,” and in research settings up to 17 “colors” (having the potential to define 131,072 2^{17} subpopulations from one 17 “color” tube) [3].

Current instrumentation provides graphical displays of cell frequency versus light intensity as a single-parameter histogram [4•]. Alternatively, the signal intensity of two parameters (“colors”) can be plotted versus cell frequency using a dot plot or a contour plot. Multiple parameters in a polychromatic study are generally evaluated using sequential two-color displays that progressively subdivide specific cell subpopulations. Typically, 10,000 to 20,000 cellular events are collected to provide sufficient numbers for meaningful data in the evaluation of subpopulations of interest. However, when cells of interest are in low abundance, collection of larger cell numbers is required [5].

Flow cytometry has emerged as a particularly useful approach to screen for the presence or absence of specific cell populations or subpopulations, the presence or absence of certain proteins that are associated with specific primary immunodeficiencies (PIDs), and biological consequences of specific immune deficiencies (Table 1) [6]. Functional flow cytometry has evolved in the evaluation of specific PIDs with applications including evaluation of the phosphorylation of intracellular signaling proteins following cell stimulation and assessment of oxidative burst (Table 1) [7, 8]. We provide below some examples of the application of flow cytometry for the study of specific categories of PIDs.

Congenital Agammaglobulinemia

Congenital agammaglobulinemia was initially described by Col. Ogden Bruton [9] in 1952 and is characterized by recurrent bacterial infections involving the sinopulmonary tract. Meningitis, encephalitis, and myositis due to enteroviral infection are also common [10]. Clinical presentation typically begins at 4–6 months of age after maternally transferred IgG has declined. Diagnosis requires the finding of very low to undetectable levels of serum immunoglobulins (IgG, IgM, and IgA) and absent specific antibody responses. Additionally, circulating B lymphocytes are absent or markedly decreased, which can be readily

Table 1 Applications of flow cytometry in the evaluation of primary immune deficiencies

Evaluate for specific cell population/subpopulation

XLA: absent B cells
 SCID: absent T cells, variable impact on B and NK cells (depending on defect)
 XLP: markedly decreased NKT cells
 ALPS: increased α/β TCR double-negative ($CD4^-/CD8^-$) T cells

Evaluate for specific protein

Cell surface
 CVID: CD19 (B cells), BAFF-R (B cells), ICOS (activated T cells)
 X-linked hyper IgM: CD40L (activated T cells)
 Autosomal recessive hyper IgM: CD40 (T cells)
 MSMD: IFN- γ R1 (monocytes), IL-12R β 1 (activated T cells)
 LAD1: CD18 (granulocytes)
 Intracellular
 XLA: BTK (monocytes, platelets)
 Wiskott-Aldrich syndrome: WASp (lymphocytes, myeloid cells)
 XLP: SAP (CD8 T cells, NK cells)
 FHL: perforin (CD8 T cells, NK cells), XIAP (lymphocytes)
 IPEX: FoxP3 ($CD4^+/CD25^+$ T cells)

Evaluate biologic effects

CVID: decreased switched memory B cells
 Omenn's syndrome (hypomorphic SCID): oligoclonal T-cell repertoire/diversity
 X-linked hyper IgM: decreased memory B cells and memory CD4 T cells

Evaluate function

MSMD: STAT1 phosphorylation (monocytes) in response to IFN- γ
 MSMD: STAT4 phosphorylation (activated T cells) in response to IL-12
 FHL: NK-cell function associated with CD107a expression (NK cells)
 CGD: DHR assay for oxidative burst (granulocytes)

ALPS autoimmune lymphoproliferative syndrome; *BTK* Bruton tyrosine kinase; *CD40L* CD40 ligand; *CGD* chronic granulomatous disease; *CVID* common variable immunodeficiency; *DHR* dihydro-rhodamine 123; *FHL* familial hemophagocytic lymphohistiocytosis; *FoxP3* forkhead box P3; *ICOS* inducible co-stimulator; *IFN* interferon; *IL* interleukin; *IPEX* immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome; *LAD1* leukocyte adhesion deficiency type 1; *MSMD* Mendelian susceptibility to mycobacterial disease; *NK* natural killer; *SAP* signaling lymphocyte activation molecule—associated protein; *SCID* severe combined immune deficiency; *STAT* signal transducer and activator of transcription; *TCR* T-cell receptor; *WASp* Wiskott-Aldrich syndrome protein; *XIAP* X-linked inhibitor of apoptosis; *XLA* X-linked agammaglobulinemia; *XLP* lymphoproliferative syndrome

determined by flow cytometry based on the lack of CD20- and/or CD19-expressing cells [11].

X-linked agammaglobulinemia (XLA) with mutations in the gene encoding Bruton tyrosine kinase (BTK) is the most common genetic etiology of congenital agammaglobulinemia. Rapid laboratory screening for this diagnosis can

be accomplished by evaluating intracellular BTK expression in monocytes or platelets using flow cytometry (XLA patients have no B cells) [12, 13]. Absence of BTK strongly suggests XLA, but detection of BTK by flow cytometry does not rule out the diagnosis, as some XLA patients express protein that is functionally defective. There are currently no flow-based methods to screen for the autosomal recessive forms of congenital agammaglobulinemia. Establishing the definitive molecular diagnosis in an agammaglobulinemic patient requires mutation analysis (see below).

Common Variable Immunodeficiency

Common variable immunodeficiency (CVID) typically presents later in life (second and third decades) and is characterized by low immunoglobulin levels and absent antibody responses, usually with relatively normal numbers of circulating B cells ($> 2\%$) [10]. Flow cytometry can be used to classify CVID patients into subgroups according to B-cell subsets, which has clinical implications [14]. Additionally, flow cytometry can screen for genetic defects associated with CVID, namely deficiency of the inducible co-stimulator (ICOS), CD19, and BAFF-R molecules [10]. ICOS deficiency can be detected by the lack of ICOS upregulation on activated T cells [15]. ICOS deficiency also leads to virtual absence of switched memory B cells and a reduction of CXCR5⁺CD4⁺ T cells [16]. BAFF-R is readily detected on normal B cells, whereas BAFF-R-deficient CVID patients have low B-cell numbers with an expanded proportion of transitional B cells [17]. CD19 deficiency is characterized by the absence of CD19 on B cells but the presence of other B-cell markers, including CD20, CD21, and surface IgM [18].

Hyper-IgM Syndromes

Hyper-IgM syndromes (HIGM) refers to a group of genetic disorders affecting molecules involved in B-cell class switch recombination and somatic hypermutation [19]. Most patients present with recurrent bacterial infections of the sinopulmonary tract with encapsulated bacteria, normal or increased IgM, and low serum levels of IgG and IgA. Mutations in seven different genes are associated with HIGM: *CD40L*, *CD40*, *AID*, *UNG*, *NEMO*, *IKBA*, and *PMS2*. Among these, *CD40L* is most frequently affected and is associated with an X-linked recessive pattern of inheritance. These patients commonly have a clinical history of opportunistic infections, given the important role of the CD40 ligand (CD40L) in T-cell function. Absent CD40L surface expression on activated CD4⁺ T cells can be demonstrated in up to 68% (41 of 61) of the patients with proven *CD40L* mutations [20]. Sensitivity can be further

enhanced to about 90% via the use of a commercially available biotinylated CD40-Ig fusion protein that only binds to a functional CD40L receptor complex. CD40 deficiency is an autosomal recessive disorder with a phenotype similar to that of CD40L deficiency and also can be screened for using flow cytometry to evaluate for the constitutive expression of CD40 on B cells, monocytes, and/or dendritic cells [21]. Flow cytometry is not useful as a screening tool for the other genetic defects associated with HIGM that are the product of B-cell defects linked directly to the B-cell class switch process.

Severe Combined Immune Deficiency

Severe combined immune deficiency (SCID), the most severe PID, presents in early infancy with recurrent opportunistic infections, chronic diarrhea, and failure to thrive [22]. Genetic etiology is varied, with all defects affecting the development or function of T cells but having variable impact on B cells and natural killer (NK) cells. Flow cytometry can help in the classification of patients based on the presence/absence of the major lymphocyte populations that point to likely genetic etiologies: T⁺B⁻NK⁻ SCID (eg, adenosine deaminase deficiency), T⁺B⁺NK⁻ SCID (eg, common γ chain (γ c), Janus kinase 3 deficiency); T⁺B⁻NK⁺ SCID (eg, RAG1/2, Artemis, ligase 4, Cernunnos deficiency), and T⁺B⁺NK⁺ SCID (eg, interleukin [IL]-7R α , CD3 chain deficiency) [23]. Additionally, in specific circumstances, such as loss-of-expression mutations of γ c (CD132), IL-7R α (CD127) or CD3 chains, flow cytometry can correctly clarify the specific genetic defect by evaluating for the absence of the specific cell surface protein. As is the case for any immunoassay, absence of a specific protein is diagnostic, but presence of the protein could still be associated with disease (based on the expression of a functionally abnormal protein yielding a false-positive screening test by flow cytometry). As previously noted, screening flow cytometry tests should be followed by specific genetic studies to define the actual mutation.

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is an X-linked recessive disorder caused by defects in the gene encoding the WAS protein (WASp) [24]. The clinical phenotype includes the classic triad of immune deficiency, thrombocytopenia, and eczema typically associated with elevated IgE levels. However, defects in the same gene can manifest as X-linked thrombocytopenia or X-linked neutropenia. Monoclonal antibodies to WASp can be used with an intracellular staining technique to screen for WASp deficiency, recognizing that some WAS patients and many X-linked

thrombocytopenia patients will demonstrate modestly decreased or even normal levels of WASp expression [25]. The evaluation of WASp expression by flow cytometry has also proven useful in the evaluation of chimerism post-hematopoietic stem cell transplantation for WAS.

Inherited Susceptibility to Mycobacterial Disease

Mendelian susceptibility to mycobacterial disease refers to a group of disorders characterized by vulnerability to infections with weakly virulent mycobacteria (environmental mycobacteria and *Mycobacterium bovis*, Bacillus Calmette-Guérin vaccine) and *Salmonella* [26]. Some patients have defects in genes of the IL-12/23–interferon (IFN)- γ pathway, and flow cytometry has proven to be a useful screening method specifically for defects in IL-12R β 1 and IFN- γ R1 [27]. About 95% of patients with IL-12R β 1 deficiency harbor mutations that abolish receptor expression, allowing easy detection by flow cytometry [27]. Among patients with autosomal recessive IFN- γ R1 defects, many demonstrate absence of surface protein expression (Fig. 1). Interestingly, an additional group of patients with autosomal dominant inheritance and a clinical phenotype that typically involves osteomyelitis demonstrate increased IFN- γ R1 expression due to overexpression of the abnormal IFN- γ R1 chain (Fig. 1) [27].

In addition to evaluating cell surface receptor expression, flow cytometric assessment of the signaling response to IFN- γ also can be performed. This screening approach evaluates intracellular signal transducer and activator of transcription-1 (STAT1) phosphorylation in monocytes following IFN- γ stimulation [28]. Identifying decreased STAT1 phosphorylation in this assay from a patient with Mendelian susceptibility to mycobacterial disease suggests that the defect involves IFN- γ R1, IFN- γ R2, or STAT1. Similarly, IL-12 signaling can be tested by evaluating the phosphorylation of STAT4 by activated T cells (necessary to induce IL-12R expression) in response to IL-12 [29].

X-Linked Lymphoproliferative Syndrome

X-linked lymphoproliferative syndrome (XLP) is a rare and severe PID that can present with the development of fatal hemophagocytosis, hypogammaglobulinemia, or lymphoma following Epstein-Barr virus infection [30]. About 60% of the familial cases are caused by mutations in *SH2D1A* (XLP1), which encodes the signaling lymphocyte activation molecule—associated protein, SAP [30]. More rarely, mutations in the X-linked inhibitor of apoptosis (*XIAP* or *BIRC4*) gene can be found in patients with XLP2 [31]. Intracellular flow cytometry testing for SAP and XIAP is a sensitive screening tool for the identification of mutation-positive patients. In recent work, flow cytometry

correctly identified all patients with mutations in these genes, similar to a previous report [32]. Additionally, in XLP1, there are very low numbers of circulating NKT (CD3⁺CD16⁺56⁺V β 24⁺V α 11⁺) cells, serving as a cue for this diagnosis [33].

Familial Hemophagocytic Lymphohistiocytosis

Patients with familial hemophagocytic lymphohistiocytosis (FHL) present with a severe disorder characterized by prolonged fevers, cytopenias, and hepatosplenomegaly, typically occurring during infancy or early childhood. Multiple genetic defects affecting granule-mediated cytotoxicity are associated with FHL, including perforin (FHL2), MUNC 13-4 (FHL3), and syntaxin-11 (FHL4) [34]. Sensitive flow-based screening assays have been developed for all three subtypes. Intracellular staining for perforin focused on cytotoxic lymphocytes (CD8 T cells, NK cells, and NKT cells) can accurately identify patients with *PRF1* (perforin) mutations as well as carriers [35]. In recent work, diminished expression of CD107a by NK cells following stimulation and degranulation has been shown to predict defects in the genes encoding MUNC 13-4 or syntaxin-11, providing a useful and rapid screening tool for FHL3 and FHL4 [36].

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is a phagocytic disorder caused by defective intracellular killing of bacterial and fungal pathogens [37]. It presents clinically with recurrent severe bacterial and fungal infections and exacerbated inflammatory reactions. Most patients (~ 65–70%) harbor X-linked recessive mutations in one component (gp91phox) of the NADPH oxidase system, while the remaining patients have an autosomal recessive defect affecting one of four additional components of this enzyme complex. The optimal screening test to clarify the diagnosis of CGD is a flow cytometric assay of NADPH oxidase activity using the intracellular dye dihydrorhodamine 123, which normally converts to a fluorescent compound following granulocyte activation (using phorbol myristate acetate) [38]. In contrast, patients with CGD show markedly decreased or absent fluorescence (little or no response to phorbol myristate acetate) in this assay. Maternal carriers of the X-linked recessive defect demonstrate two populations of cells corresponding to the random inactivation of the healthy or defective X chromosome.

Leukocyte Adhesion Deficiency Type 1

Leukocyte adhesion deficiency type 1 is caused by defects in the gene that encodes CD18 and results in decreased or

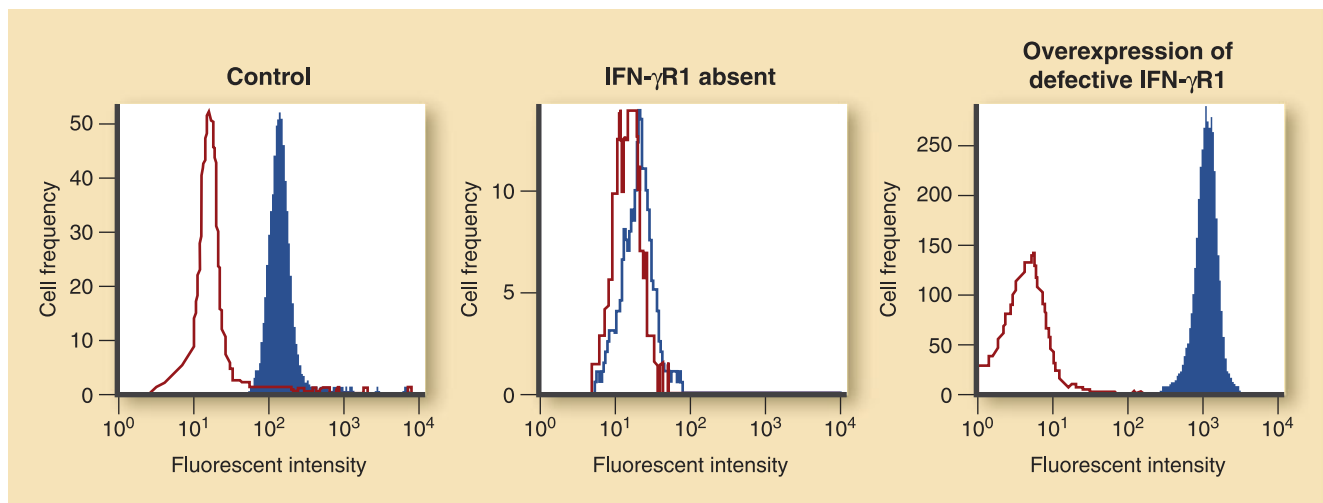


Fig. 1 IFN- γ R1 defects associated with Mendelian susceptibility to mycobacterial disease. Shown are IFN- γ R1 expression on a healthy control (*left panel*), one patient with an autosomal recessive mutation

leading to absent receptor expression (*middle panel*), and one patient with a dominant mutation causing over-accumulation of defective receptor on the cell surface (*right panel*). IFN—interferon

absent expression of the three heterodimeric $\beta 2$ integrin receptors (CD11a/CD18, CD11b/CD18, CD11c/CD18). Patients present with recurrent bacterial and fungal infections of varying severity [39]. The optimal screening test to diagnose this disorder relies on flow cytometry to test for CD18 (and CD11a, CD11b, and CD11c) expression on granulocytes. Leukocyte adhesion deficiency type 1 patients typically show low (< 10%) to absent CD18 expression accompanied by a marked peripheral leukocytosis. In reality, there is functional neutropenia that reflects the inability of the granulocytes to migrate from the intravascular space to sites of infection.

Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Inheritance Syndrome

Immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome (IPEX) typically presents during infancy with the clinical triad of severe enteropathy, endocrinopathies (diabetes or thyroiditis), and eczematous dermatitis [40]. Most IPEX patients harbor mutations in *FOXP3*, encoding the winged helix transcription factor forkhead box P3 (FoxP3) that is crucial for the development and function of T-regulatory cells (Tregs). Detection of circulating FoxP3-expressing CD4⁺/CD25⁺ T cells can be performed using a combination of cell surface and intracellular flow cytometry to identify the FoxP3⁺ Tregs. About 80% of IPEX patients demonstrate low levels of FoxP3⁺ Tregs compared with healthy controls (Torgerson, personal communication), and this flow-based assay serves as a simple screening test for IPEX. One caveat is the timing between blood collection and analysis, which should not exceed 36 h due to a significant time-dependent

spontaneous loss of FoxP3⁺ Tregs (Torgerson, personal communication).

Autoimmune Lymphoproliferative Syndrome

Autoimmune lymphoproliferative syndrome is a genetic disorder of lymphocyte apoptosis characterized by lymphadenopathy and/or splenomegaly, multilineage autoimmune cytopenias, and propensity to develop B-cell lymphomas [41]. Most cases (65%) are caused by mutations in the gene encoding Fas (*FAS/TNFRSF6*), while a minority of patients have other genetic defects, including mutations in genes encoding Fas ligand (*FASLG*) and caspase-10 (*CASP10*). A hallmark and required immunophenotypic finding is the presence of elevated levels of circulating T-cell receptor (TCR) α/β expressing CD4⁺CD8⁺ T cells, referred to as *double-negative T cells* (DNTs) [42]. This population must be clearly distinguished from TCR γ/δ DNTs. In addition, evaluation of the Fas receptor (CD95) may demonstrate moderate reductions (~ 50%) of Fas expression associated with a single (heterozygous) null mutation affecting the extracellular region of the protein (unpublished data).

Molecular Diagnosis

Mutation Analysis

In 1986, automation of the Sanger method of DNA sequencing revolutionized modern molecular biology; it remains the mainstay in DNA sequencing for clinical mutation analysis at this juncture [43]. This method uses

DNA polymerase to transcribe specific regions of the template DNA under controlled conditions with labeled dideoxynucleotides (ddNTPs) used to randomly terminate chain elongation. As a ddNTP contains no 3'-hydroxyl group, a newly synthesized DNA strand that incorporates a ddNTP cannot be extended further, so termination occurs specifically at the position at which the ddNTP was incorporated instead of the corresponding deoxyribonucleotide. In standard clinical assays, each of the ddNTPs (ddATP, ddCTP, ddGTP, ddTTP) is labeled with a different fluorescent dye, with each emitting a different (defined) wavelength. The result is a mixture of fragments, all having the same 5' end, with one of the four fluorescently labeled ddNTPs at the 3' end. This mixture is fractionated according to length (size) by automated capillary electrophoresis using a denaturing polymer gel. An electronically derived pattern of peaks, known as a *chromatogram*, shows the distribution of ddNTPs in the newly synthesized DNA, and the DNA sequence of the original template is directly inferred from these data (Fig. 2).

The standard approach for mutation analysis in PIDs starts with the use of genomic DNA isolated from any source of patient cells [44]. This is satisfactory to establish a specific disease-causing mutation in most patients (Fig. 2), but practicing clinicians should be aware of situations that can result in a false negative (no mutation found). First, deletion of entire exons or larger genomic regions in somatic (non-sex) chromosomes can result in failure to amplify an abnormal allele, resulting in spuriously normal results. If the clinical suspicion is strong, one approach to overcome this potential problem is to have the laboratory evaluate gene or exon copy number by real time polymerase chain reaction or comparative genome hybridization assays. Second, there may be mutations in non-exonic DNA regions that affect gene expression. These areas are usually not examined in routine genomic mutation analysis. This could include splice sites or intronic regulatory elements and could lead to a false-negative

result (no mutation found). Lastly, an additional source of error can result from a somatic mutation affecting only a subset of cells, such that the genomic DNA tested could be derived primarily from unaffected cells and yield a false-negative result. In these various situations, additional evaluation using alternative molecular methods is indicated. This often requires moving to cDNA for sequencing and/or evaluating the protein product (immunoblot) as well as performing functional testing when this is available.

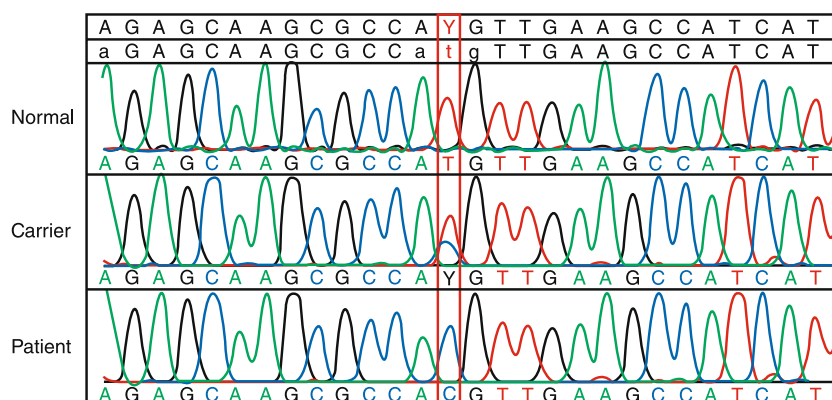
Another source of potential difficulty in interpreting mutation testing is the finding of novel DNA variation in a particular gene not previously associated with disease. In this situation, the determination of a causal role for the mutation associated with disease would require additional functional testing and/or bioinformatics analyses [45]. This should also include a review of possible known polymorphisms in the gene and recognition that there is also the possibility of private polymorphisms that would not be the cause of disease.

It is likely that next-generation DNA sequencing technologies applied more broadly in the near future will have an even greater impact on diagnosing and managing human disease [46]. Although it is currently primarily used in research settings, this rapidly moving field will likely move into the clinical laboratory, providing either whole exon (exomic) or complete genome sequencing for the purpose of a genetic evaluation.

T-cell Receptor Excision Circle Quantification

TRECs are a byproduct of the editing of the TCR genes during T-cell differentiation and maturation and persist within the cell as extragenomic pieces of DNA. The intracellular TRECs are diluted as the cell proliferates, such that naïve T cells present with relatively high TREC levels, whereas antigen-experienced (memory) T cells have low TREC levels [47]. In the setting of PIDs, TREC evaluation can be used as a diagnostic confirmation of low

Fig. 2 Mutation analysis in severe combined immune deficiency. Chromatograms demonstrate wild-type (normal) DNA (*upper panel*), a maternal carrier for the γ c chain mutation (two peaks, normal and abnormal nucleotide, *middle panel*), and the patient (single peak different from wild-type, *lower panel*)



thymic output found in DiGeorge syndrome and hypomorphic forms of SCID, including Omenn's syndrome, as well as to monitor thymic output during immune reconstitution after hematopoietic stem cell and thymus transplantation.

Additionally, the quantification of TRECs on a blood spot obtained from Guthrie cards is being piloted as a neonatal screening tool for severe T-cell immunodeficiency in Wisconsin and Massachusetts [48]. The finding of low TREC levels in neonates should prompt immediate follow-up and additional evaluation. It is hoped that the wider application of TRECs as part of neonatal screening will decrease the mortality associated with SCID and other severe T-cell deficiencies by facilitating earlier curative therapy.

T-cell Repertoire/Diversity Evaluation

Circulating peripheral blood T cells have a diverse repertoire and express the majority of the 24 TCR V β chain families. Within each V β family, there is further variability, reflecting different lengths of the CDR3 region. The CDR3 diversity and distribution of V β families can be assessed by polymerase chain reaction and flow cytometry, respectively [7]. In settings in which there is an oligoclonal T-cell population, such as is found in Omenn's and atypical DiGeorge syndromes, a very distorted (non-Gaussian) distribution with abnormal frequency of V β families is observed and provides important information to confirm the specific diagnosis [49].

Conclusions

Mutation analysis and flow cytometry have emerged as critical tools in the evaluation and diagnosis of PIDs. The application of flow cytometry provides rapid results and in many cases clarifies the likely diagnosis, directs further immunologic studies, and/or can be linked to clinical phenotype. Mutation analysis provides the most definitive means of diagnosis in a setting in which new genes associated with PIDs continue to be defined [50]. It is highly likely that in the near future, next-generation sequencing will be the mainstay of the genetic diagnosis of PIDs; however, flow cytometry and functional testing will remain important adjuncts in the evaluation of patients suspected of having immune deficiencies.

Acknowledgment Preparation of this manuscript was supported by the National Institutes of Health Intramural Research Program of the National Institutes of Health Clinical Center.

Disclosure No potential conflicts of interest relevant to this article were reported.

References

Papers of particular interest, published recently have been highlighted as:

- Of importance

1. Givan AL: Flow cytometry: an introduction. *Methods Mol Biol* 2004, 263:1–32.
2. Loken MR, Brosnan JM, Bach BA, Ault KA: Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990, 11:453–459.
3. Perfetto SP, Chattopadhyay PK, Roederer M: Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004, 4:648–655.
4. Fleisher TA, Oliveira JB: Flow cytometry. In *Clinical Immunology: Principles and Practice*, edn 3. Edited by Rich RR, Shearer WT, Schroeder HW Jr, et al. Philadelphia, PA: Mosby Elsevier; 2008:1435–1446. *This chapter provides a more in-depth discussion of the principles of flow cytometry as well as the applications of this technology.*
5. Gratama JW, Kraan J, Keeney M, et al.: Validation of the single-platform ISHAGE method for CD34(+) hematopoietic stem and progenitor cell enumeration in an international multicenter study. *Cytotherapy* 2003, 5:55–65.
6. Oliveira JB, Notarangelo LD, Fleisher TA: Applications of flow cytometry for the study of primary immune deficiencies. *Curr Opin Allergy Clin Immunol* 2008, 8:499–509.
7. Fleisher TA, Oliveira JB: Functional and molecular evaluation of lymphocytes. *J Allergy Clin Immunol* 2004, 114:227–234.
8. Bleesing JJ, Fleisher TA: Cell function-based flow cytometry. *Semin Hematol* 2001, 38:169–178.
9. Bruton OC: Agammaglobulinemia. *Pediatrics* 1952, 9:722–728.
10. Yong PF, Chee R, Grimbacher B: Hypogammaglobulinaemia. *Immunol Allergy Clin North Am* 2008, 28:691–713.
11. Conley ME, Dobbs AK, Farmer DM, et al.: Primary B cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol* 2009, 27:199–227.
12. Futatani T, Watanabe C, Baba Y, et al.: Bruton's tyrosine kinase is present in normal platelets and its absence identifies patients with X-linked agammaglobulinaemia and carrier females. *Br J Haematol* 2001, 114:141–149.
13. Futatani T, Miyawaki T, Tsukada S, et al.: Deficient expression of Bruton's tyrosine kinase in monocytes from X-linked agammaglobulinemia as evaluated by a flow cytometric analysis and its clinical application to carrier detection. *Blood* 1998, 91:595–602.
14. Wehr C, Kivioja T, Schmitt C, et al.: The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* 2008, 111:77–85.
15. Grimbacher B, Hutloff A, Schlesier M, et al.: Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nat Immunol* 2003, 4:261–268.
16. Bossaller L, Burger J, Draeger R, et al.: ICOS deficiency is associated with a severe reduction of CXCR5+ CD4 germinal center Th cells. *J Immunol* 2006, 177:4927–4932.
17. Warnatz K, Salzer U, Rizzi M, et al.: B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. *Proc Natl Acad Sci U S A* 2009, 106:13945–13950.
18. van Zelm MC, Reisli I, van der Burg M, et al.: An antibody-deficiency syndrome due to mutations in the CD19 gene. *N Engl J Med* 2006, 354:1901–1912.
19. Gulino AV, Notarangelo LD: Hyper IgM syndromes. *Curr Opin Rheumatol* 2003, 15:422–429.

20. Lee WI, Torgerson TR, Schumacher MJ, et al.: Molecular analysis of a large cohort of patients with the hyper immunoglobulin M (IgM) syndrome. *Blood* 2005, 105:1881–1890.
21. Ferrari S, Giliani S, Insalaco A, et al.: Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* 2001, 98:12614–12619.
22. Puck JM: Severe combined immunodeficiency: new advances in diagnosis and treatment. *Immunol Res* 2007, 38:64–67.
23. Geha RS, Notarangelo LD, Casanova JL, et al.: Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. *J Allergy Clin Immunol* 2007, 120:776–794.
24. Notarangelo LD, Miao CH, Ochs HD: Wiskott-Aldrich syndrome. *Curr Opin Hematol* 2008, 15:30–36.
25. Kawai S, Minegishi M, Ohashi Y, et al.: Flow cytometric determination of intracytoplasmic Wiskott-Aldrich syndrome protein in peripheral blood lymphocyte subpopulations. *J Immunol Methods* 2002, 260:195–205.
26. Bustamante J, Boisson-Dupuis S, Jouanguy E, et al.: Novel primary immunodeficiencies revealed by the investigation of paediatric infectious diseases. *Curr Opin Immunol* 2008, 20:39–48.
27. Filipe-Santos O, Bustamante J, Chagier A, et al.: Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. *Semin Immunol* 2006, 18:347–361.
28. Fleisher TA, Dorman SE, Anderson JA, et al.: Detection of intracellular phosphorylated STAT-1 by flow cytometry. *Clin Immunol* 1999, 90:425–430.
29. Uzel G, Frucht DM, Fleisher TA, Holland SM: Detection of intracellular phosphorylated STAT-4 by flow cytometry. *Clin Immunol* 2001, 100:270–276.
30. Nichols KE, Ma CS, Cannons JL, et al.: Molecular and cellular pathogenesis of X-linked lymphoproliferative disease. *Immunol Rev* 2005, 203:180–199.
31. Rigaud S, Fondaneche MC, Lambert N, et al.: XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* 2006, 444:110–114.
32. Zhu Q, Ochs H: Rapid flow cytometric screening for SAP and XIAP defects in patients with a phenotype of X-linked lymphoproliferative disease (XLP). *Clin Immunol* 2008, 127:s73.
33. Tabata Y, Villanueva J, Lee SM, et al.: Rapid detection of intracellular SH2D1A protein in cytotoxic lymphocytes from patients with X-linked lymphoproliferative disease and their family members. *Blood* 2005, 105:3066–3071.
34. Filipovich AH: Hemophagocytic lymphohistiocytosis and other hemophagocytic disorders. *Immunol Allergy Clin North Am* 2008, 28:293–313, viii.
35. Kogawa K, Lee SM, Villanueva J, et al.: Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. *Blood* 2002, 99:61–66.
36. Marcenaro S, Gallo F, Martini S, et al.: Analysis of natural killer-cell function in familial hemophagocytic lymphohistiocytosis (FHL): defective CD107a surface expression heralds Munc13-4 defect and discriminates between genetic subtypes of the disease. *Blood* 2006, 108:2316–2323.
37. Rosenzweig SD, Holland SM: Phagocyte immunodeficiencies and their infections. *J Allergy Clin Immunol* 2004, 113:620–626.
38. Jirapongsananuruk O, Malech HL, Kuhns DB, et al.: Diagnostic paradigm for evaluation of male patients with chronic granulomatous disease, based on the dihydrorhodamine 123 assay. *J Allergy Clin Immunol* 2003, 111:374–379.
39. Etzioni A: Defects in the leukocyte adhesion cascade. *Clin Rev Allergy Immunol* 2009, 38:54–60.
40. Torgerson TR, Ochs HD: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. *J Allergy Clin Immunol* 2007, 120:744–750.
41. Fleisher TA, Oliveira JB: Autoimmune lymphoproliferative syndrome. *Isr Med Assoc J* 2005, 7:758–761.
42. Bleesing JJ, Brown MR, Straus SE, et al.: Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. *Blood* 2001, 98:2466–2473.
43. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 1977, 74:5463–5467.
44. Hsu AP, Fleisher TA, Niemela JE: Mutation analysis in primary immunodeficiency diseases: case studies. *Curr Opin Allergy Clin Immunol* 2009, 9:517–524.
45. Thusberg J, Vihinen M: Pathogenic or not? And if so, then how? Studying the effects of missense mutations using bioinformatics methods. *Hum Mutat* 2009, 30:703–714.
46. Metzker ML: Sequencing technologies—the next generation. *Nat Rev Genet* 2010, 11:31–46.
47. Dion ML, Sekaly RP, Cheynier R: Estimating thymic function through quantification of T-cell receptor excision circles. *Methods Mol Biol* 2007, 380:197–213.
48. Puck JM: Population-based newborn screening for severe combined immunodeficiency: steps toward implementation. *J Allergy Clin Immunol* 2007, 120:760–768.
49. Villa A, Notarangelo LD, Roifman CM: Omenn syndrome: inflammation in leaky severe combined immunodeficiency. *J Allergy Clin Immunol* 2008, 122:1082–1086.
50. • Notarangelo LD, Fischer A, Geha RS, et al.: Primary immunodeficiencies: 2009 update. *J Allergy Clin Immunol* 2009, 124:1161–1178. *This article provides in tabular form an outline of all currently recognized (at the time of publication) PIDs, including the specific molecular defect.*