Role of Flow Cytometry in the Diagnosis and Monitoring of Primary Immunodeficiency Disease

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Over the past 3 decades, flow cytometry has emerged as an invaluable technology in clinical laboratories and has contributed significantly to the understanding and evaluation (monitoring) of the immune system. The unparalleled ability to simultaneously identify characteristic physical cell properties, cell functions, and numerous gene products at rates of thousands of cells per second has resulted in the development of a large repertoire of diagnostic, prognostic, and monitoring assays. There currently are more than 120 recognized primary immune diseases that have been classified and whose underlying genetic defect is known. The diagnosis of many of these disorders is supported strongly by a wide variety of flow cytometry applications. Abnormalities detected by flow cytometry can be grouped broadly as having (1) relative or absolute decrease in a specific subset or subsets, (2) loss or abnormal expression of a specific cell-associated marker or markers, and (3) loss or abnormal function. This article is organized according to the recent classification of primary immunodeficiencies published by the International Union of Immunological Societies Primary Immunodeficiency meeting \cite{1}. The diseases have been classified into eight groups, which are listed at the beginning of each section. After each list, individual diseases that are amenable to assessment by flow cytometry are reviewed with a brief clinical description and a discussion of the appropriate flow cytometry application. Where appropriate, figures and brief summaries of specific procedures are provided.

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Primary immunodeficiency classification

Beginning in 1970, the World Health Organization convened a group of experts whose role was to classify (unified nomenclature) and define the primary immunodeficiency diseases (PIDs) [2,3]. The World Health Organization sponsored several more meetings of experts in PIDs until recently, when the sponsorship of the committee was assumed by the International Union of Immunological Societies and the Jeffrey Modell Foundation [4] and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health [1,5]. Between 2001 and 2006, the human genome project has led to the identification of the molecular basis for several PIDs. At the most recent classification meeting held in Budapest, Hungary, more than 120 PIDs with known molecular etiologies were reviewed and classified into eight categories. New diseases were included and the nomenclature of some PIDs was amended to describe more closely the nature of the underlying genetic defect [1]. The newest classification includes more than 120 defined diseases classified into eight categories, some of which are more amenable to flow cytometric evaluation than others (summarized in Table 1).

The majority of the PIDs listed in group I (Box 1) lead to a peripheral blood abnormality that can be detected by flow cytometry. The routine panel (described later) provides a useful and fruitful tool for the screening

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<th>Group</th>
<th>Category</th>
<th>Flow application</th>
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<tr>
<td>Group I</td>
<td>Combined T- and B-cell immunodeficiencies</td>
<td>Several PIDs contained in this group are characterized directly by flow cytometry</td>
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<tr>
<td>Group II</td>
<td>Predominantly antibody deficiencies</td>
<td>Absence of B cells or of B-cell markers assessed readily</td>
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<td>Group III</td>
<td>Other well-defined immunodeficiency syndromes</td>
<td>Only a few PIDs in this group can be characterized</td>
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<td>Group IV</td>
<td>Diseases of immunodysregulation</td>
<td>A few very specific abnormalities can be characterized</td>
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<td>Group V</td>
<td>Congenital defects of phagocyte number, function, or both</td>
<td>Several specific procedures virtually diagnostic of the specific PID</td>
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<td>Group VI</td>
<td>Defects in innate immunity</td>
<td>Limited applications involving flow cytometry&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Group VII</td>
<td>Autoinflammatory disorders</td>
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<td>Group VIII</td>
<td>Complement deficiencies</td>
<td>Limited applications involving flow cytometry&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> Not discussed further.

of group I diseases. Particular abnormalities may be specific for a known genetic defect or at least can help direct the search to only a few candidate genes.

**Routine immunophenotyping panel for the identification of subset abnormalities in primary immunodeficiency disease**

Routine immunophenotyping is designed to measure qualitative and quantitative abnormalities in the major peripheral blood lymphocyte subsets
and is an extremely valuable procedure for the screening of several PIDs. My laboratory currently uses the panel in Table 2 to measure the relative and absolute number of B cells (CD19⁺), T cells (CD3⁺), T-helper cells (Th, CD3⁺, and CD4⁺), T-cytotoxic cells (Tc, CD3⁺, and CD8⁺), natural killer (NK) cells (CD3 negative and CD16⁺ or CD56⁺), and activated T cells (CD3⁺ and HLA-DR⁺). The CD3, HLA-DR tube assesses activated T cells but also serves as a control for the measurement of B cells (CD3 negative and bright HLA-DR⁺) and HLA-DR⁺ non-B, non-T cells. Currently, each tube contains up to four fluorochrome-conjugated antibodies; however, the same information can, and successfully has been, obtained using two-, three-, or five-color combinations (see current panel in Table 2). Several commercially available monoclonal antibody products are available for the detection of the subsets (described previously); however, it is a laboratory’s responsibility to ensure that the equipment and the reagents are maintained properly and quality controlled before their implementation.

Historically, absolute lymphocyte subset counts have been derived as the product of the absolute lymphocyte count generated by an automated hematology instrument and the individual lymphocyte subset percentage obtained by flow cytometry (dual-platform method). It rapidly is becoming apparent that absolute counts derived directly from the flow cytometer (single-platform assays) combined with the use of CD45 versus right-angle light scatter lymphocyte gating is more precise and more robust than the traditional dual-platform methods [6,7]. Although the majority of these developments were validated in populations of patients who were HIV-1 infected, the recommendations are appropriate for immunophenotyping patients suspected of having PID. The recommendation for the use of CD45 expression levels versus right-angle light scatter properties for lymphocyte gating [8] is appropriate particularly for the analysis of PIDs associated with a profound lymphopenia (see comparison versus traditional light scatter gating in Fig. 1).

\[ \text{Th} - \text{Tc} - B^+NK^+X\text{-linked severe combined immunodeficiency: common gamma chain} \]

X-linked SCID is the variant of the SCID syndromes that is observed most commonly [8]. It is caused by mutations in the gene encoding the IL-2Rγ, also referred to as the common gamma chain (γc) or CD132,

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<td>#1</td>
<td>CD3</td>
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and leads to a failure in the development of mature circulating T cells and NK cells [9,10] (exemplified in Fig. 2). The common gamma chain (\(\gamma_c/CD132\)) also is a component of the IL-4, IL-7, IL-9, IL-15, and IL-21 receptors [11]. B cells from patients who have X-linked SCID are abnormal, do not mature, and fail to undergo class switch recombination [12]. Anti-\(\gamma_c\) monoclonal antibodies are available directly fluorescinated from several companies and are used successfully to detect absent or abnormal protein expression [13,14].

\[Th^{-}Tc^{-}B^{+}NK^{-}, \text{autosomal severe combined immunodeficiency (JAK3)}\]

An autosomal form of SCID that is clinically and immunophenotypically indistinguishable from the X-linked form is associated with mutations in gene encoding the JAK3, reviewed by Roberts and colleagues [15]. After successful bone marrow transplantation in patients not undergoing pretransplant myeloablative therapy, B cell and NK cells fail to function normally in X-linked and autosomal JAK3 SCID. This is because B cells and NK cells of the donor do not engraft, and the abnormalities related to IL-4 and IL-21 cytokine receptors in the B cells and abnormal IL-15R in NK cells prevent normal functioning even in the presence of normal T-cell help by donor-derived T cells [12]. Autosomally inherited JAK3 mutations represents the fourth most common genetic defect is SCID [12].

\[Th^{-}Tc^{-}B^{+}NK^{-}(CD45)\]

Patients who have mutations in the gene encoding CD45 have significantly reduced percentages and absolute numbers of T cells and NK cells
In addition to the abnormal subset representation observed, the level of CD45 expression on the surface of the hematopoietic cells is absent or severely reduced. Th–Te–B+NK+(interleukin-7 receptor alpha and CD3δ)

Mutations in the IL-7Ra gene represent the third most common cause of SCID [17] and [12]. These patients have a preponderance of B cells with relatively normal levels of NK cells. Although rare, mutations in the delta chain of the CD3 receptor also are described as causing clinical SCID.
with a profound deficiency of CD3+, CD4+, and CD8+ circulating T cells with a virtual absence of gamma/delta T-cell receptor (TCR)-positive T cells [18].

\[ \text{Th}−\text{Tc}−\text{B}−\text{NK+} (\text{RAG1, RAG2, LIG IV, and Artemis}) \]

The absence of (or abnormally low) circulating B and T cells with the presence of circulating NK cells (Fig. 3) forms another distinct group of PID. This abnormal phenotype is the result of mutations in several different genes involved in the process of specific antigen-receptor formation on B and T cells. The large variability in the antigen recognition motifs of B-cell receptors and TCRs is generated by the process of somatic V (D) J recombination in the TCR and B-cell receptor genes. This highly regulated molecular event is essential for the maturation of circulating peripheral blood B cells and T cells. RAG1 and RAG2 interact with the appropriate sequences in the V and J (and sometimes D) regions to induce a double-strand break. The recombination (repair) process then is effected by components of the nonhomologous end-joining machinery, which is composed of at least six proteins, Ku70, Ku80, DNA-PKcs, Artemis, DNA ligase IV (LIG IV), and Xrcc4 [19]. Mutations in genes inducing the double-strand breaks (ie, RAG1 and RAG2) [20] and mutations in the genes encoding the proteins responsible for the repair of the double-strand breaks (Artemis [21] and LIG IV [22,23]) lead to SCID disease with no or very low levels of T and B cells. In Europe, the V (D) J recombination defects are responsible for approximately 20% of SCID cases [24], whereas in the United States, the V (D) J recombination defects are less common, representing less than 3% of the genetic abnormalities of patients who have SCIDs [12].

\[ \text{Th}−\text{Tc}−\text{B}−\text{NK−} (\text{adenosine deaminase deficiency}) \]

Patients who have severe lymphopenia (<500 cells/μL) and a virtual absence of all lymphocyte subsets should be suspected of harboring mutations in the ADA gene. Patients who have ADA deficiency account for approximately 15% to 20% of all patients who have SCID in Europe and the United States [24–26]. In addition to a more severe lymphopenia than other forms of SCID, patients who have ADA deficiency exhibit other distinguishing features, including multiple skeletal abnormalities on radiograph [12].

\[ \text{Th}+\text{Tc}+\text{B}+\text{NK−} (\text{interleukin-2 receptor/interleukin-15 receptor beta}) \]

A 17-month-old boy who had a complete absence of NK cells in the peripheral circulation, decreased T cells, and normal levels of B cells has been described [14]. Using flow cytometry with monoclonal antibodies specific for the IL-15R (anti–IL-2R/IL-15Rβ, Santa Cruz Biotechnology, Santa Cruz, California), it was observed that the IL-2R/IL-15Rβ expression
levels were less than 10% of normal. The messenger RNA (mRNA) levels for IL-2R/IL-15Rβ also were detected at less than 10% of the control. No mutations were detected; however, the sensitivities of the techniques used were not 100%.

*Omenn syndrome*

Patients who have hypomorphic mutations (leading to some expression of an abnormal gene product) in Artemis [21], RAG1 or RAG2 [27], or IL-7R [28] can develop a SCID variant disease referred to as the Omenn syndrome. Clinically, these patients present with erythematous rash (98%),
hepatosplenomegaly (88%), lymphadenopathy (80%), and recurrent infections (reviewed by Aleman and colleagues) [29]. In addition to frequent infections, patients often have high serum IgE, and an elevated white blood cell count as a result of eosinophilia or lymphocytosis. Patients who have Omenn syndrome can have normal to elevated levels of circulating T-cell numbers but they are abnormally activated (very high HLA-DR expression, activated T-cell phenotype [Fig. 4]) and can have a skewed TCR repertoire (Figs. 5 and 6).

T-cell receptor V beta repertoire analysis in CD4 and CD8 T-cell subsets

My colleagues and I have developed a TCR V beta repertoire analysis test in CD4 and CD8 T-cell subsets by slightly modifying a commercial kit (Beckman Coulter, Hialeah, Florida). Each tube contains three different V
beta monoclonal antibodies (fluorescein isothiocyanate [FITC], phycoerythrin [PE], and FITC + PE), CD3–peridinin chlorophyll protein (PerCP), and CD8–allophycocyanine (APC) (ie, four colors). This combination of monoclonal antibodies and fluorochromes allows measuring the relative representation of three individual TCR V beta families in CD3+CD8− T cells and CD3+CD8+ T cells (primarily CD4+) in each tube for a total of 24 different TCR V beta families (illustrated in Fig. 5). This panel is
useful in supporting the diagnosis of Omenn syndrome (described previously) (see Fig. 4) and is reported as useful in assessing the degree of immunologic compromise in patients who have DiGeorge syndrome [30].

Summary of severe combined immunodeficiency

To date, there are 10 genes (ADA, CD132, RAG1, RAG2, LIG4, Artemis, JAK3, CD3δ, CD45, and IL-7R) known to be associated with SCID.
disease. As described previously, many of the mutations lead to characteristic abnormalities in the development of T cells, B cells, and NK cells. A routine flow cytometric assay for the measurement of the relevant proportions and absolute numbers of the major lymphocyte subsets can provide valuable clues to the underlying genetic abnormality in a fast and relatively inexpensive manner.

Hyper-IgM syndromes: CD40 ligand and CD40 receptor deficiencies (group I)

The HIGM syndromes with immunoglobulin class-switch defects (previously classified as the HIGM syndromes 1, 2, and so forth) have been reclassified according to their genetic defect and now are split between group I (combined immunodeficiencies, CD40 ligand, and CD40 receptor) and group II (primarily antibody deficiency groups, NF-κB essential modulator NEMO, uracil-DNA glycosylase [UNG], and activation-induced cytidine deaminase [AICDA]) [1,31]. These syndromes are characterized by low levels of serum IgG and IgA with normal to elevated IgM, defective immunoglobulin class switch recombination and absence of immunoglobulin gene somatic hypermutation [31]. Patients who have mutations in the genes encoding the CD40 receptor or the CD40 ligand tend to have cellular and humoral immunologic abnormalities and more severe clinical disease. In a recent review of 130 patients who had HIGM, 75% of the individuals were found to have a mutation in the X-linked gene encoding CD40 ligand [31]. The CD40 ligand, expressed preferentially on the surface of activated CD4+ T cells, binds to the CD40 receptor of B cells and, along with the appropriate cytokines, induces their proliferation, differentiation, and immunoglobulin class switching of the immunoglobulin genes. CD154 can be measured reliably only after a potent in vitro activation step, which has led to the development of a function-based flow cytometry assay (discussed later) [32].

Measurement of CD40 ligand on in vitro–activated T-helper cells and CD40 receptor on B cells

A function-based flow cytometry procedure for the screening diagnosis of patients who have XHIM and carriers suspected of having the disease was developed in my laboratory [32]. Briefly, whole blood is stimulated with phorbol ester and a calcium ionophore for 4 hours, then labeled with a panel of monoclonal antibodies, including CD3, CD8, CD40 ligand (TRAP-10 clone conjugated with phycoerytherin) (Pharmingen, San Diego, California), and CD69. Samples then are analyzed using a novel gating strategy (see Fig. 6). Abnormal CD40 ligand expression has been detected in all of my patients who have XHIM (to date), their carrier mothers (when available), and in one of three patients who have common variable immunodeficiency (CVID) (see Fig. 6 for an example of an XHIM in patient and...
carrier). It must be cautioned that others have reported that some of the XHIM mutations might be missed if their detection relied exclusively on monoclonal antibodies [31]. The flow cytometry procedure is relatively expensive (especially if laboratories do not receive a significant volume of request); however, most laboratories with a moderate amount of flow cytometry experience could perform this assay [33].

Mutations in the gene encoding the CD40 receptor (HIGM3) on B cells (not surprisingly) also lead to a clinical phenotype of the HIGM syndrome. Abnormal expression of the CD40 receptor is detected by gating on B cells (CD19 or CD20) and measuring the percentage of CD40-positive events (greater than 90% of B cells express CD40 [Maurice O’Gorman, PhD, unpublished result, 2006]). The latter form of autosomal recessive HIGM syndrome is rare [34].

Abnormal HLA-DR expression (Omenn syndrome and major histocompatibility complex II deficiency)

Elevated HLA-DR+ T cells can provide a clue to the diagnosis of a PID in at least two well characterized scenarios: (1) maternally engrafted T cells in patients who have SCID and (2) patients who have hypomorphic mutations in RAG1 or RAG2, Artemis, or IL-7α genes (patients who have Omenn syndrome [discussed previously]). If maternal engraftment is ruled out, the presence of TCR V beta repertoire abnormalities combined with elevated surface expression of HLA-DR (Fig. 7) is suggestive of a diagnosis of Omenn syndrome (see Fig. 4).

Mutations in genes encoding the class II transcription activation factor and the regulatory factor X proteins (RFX5, RFXAP, and RFXANK) cause abnormal MHC class II expression on the surface of B cells, NK cells (reviewed by Masternak and colleagues [35]), and activated T cells. In my colleagues’ and my routine immunophenotyping panel, B cells that normally express high levels of HLA-DR on their surface are negative and patients often have abnormally low percentages of CD4+ T cells, presumably because of abnormal selection in the thymus (Fig. 8).

Decreased CD8+ T cells (transporter associated with antigen processing 1 and 2 deficiency and zeta-associated protein of 70 kd deficiency)

Mutations in the gene encoding the TAP2 subunit of the peptide TAP lead to a failure of MHC class I to be loaded with peptide [36], which in turn leads to a failure of class I expression on the cell surface. Lack of class I expression leads to abnormal selection of CD8+ T cells in the thymus. Patients who have TAP deficiency are reported to have decreased numbers of alpha/beta TCR-positive CD8 T cells, a higher proportion of gamma/delta-positive CD8+ T cells, and normal levels of NK cells (both CD3-CD56/CD16+ and CD3-CD8+ NK cells); however, NK function is abnormal [36]. This disease also is referred to as bare lymphocyte syndrome.
Fig. 7. Flow cytometry procedure for the screening diagnosis of XHIM, now referred to as CD40-ligand deficiency. Heparinized peripheral blood is activated with an optimal concentration of PMA and the calcium ionophore ionomycin (Sigma Chemical, St. Louis, Missouri). After 4 hours at 37°C, samples are washed and labeled with CD3-PerCP, CD40-ligand-PE, and CD8-APC (or FITC). CD40 ligand is expressed primarily on activated CD4+ T cells; however, the in vitro stimulation process results in the modulation of CD4 off of the cell surface. Therefore, the CD4+ T cells are analyzed by a negative gating strategy (i.e., by drawing a region around the CD3+CD8− T cells) (bottom left dot plot). Each histogram in the right-hand column illustrates the level of CD40 ligand expressed (filled curves) on T-helper cells express gated as described. The stippled curve in each histogram represents the level of fluorescence generated on CD3+CD8− events by an isotype-matched control antibody and the filled peaks represent the fluorescence generated on the same events by CD40-ligand-PE. Note that the patient’s T-helper cells express very little CD40 ligand (<5%) (middle histogram, right-hand column), whereas his mother’s cells express only 60% (bottom histogram, right-hand column). These results are consistent with a diagnosis of XHIM in the patient and X-linked carrier status in the mother. Not depicted in the figure are the results showing normal CD69 expression on the in vitro activated CD3+ T cells on samples from the patient, the mother, and the control, eliminating the possibility of a nonspecific in vitro activation abnormality.
Patients who have mutations in ZAP-70 exhibit an absence of circulating CD8+ T cells and their CD4+ T cells fail to respond to TCR signaling in vitro [37]. Patients who have ZAP-70 mutations have abnormal TCR-mediated activation [38], resulting in severely depressed T-cell function and a clinical presentation similar to that of other patients who have SCID [37,39].

Decreased CD4+ T cells

The idiopathic CD4+ T-lymphopenia syndrome is characterized by low CD4 counts (<300 cell/μL) or low CD4% (<20%) on at least two occasions with evidence of opportunistic infections and rigorous exclusion of...
all other known primary and acquired immunodeficiency states (eg, HIV infection) [40]. This syndrome is reported in children and adults and in patients who have Down syndrome [41].

There is a report of immunodeficiency associated with homozygous caspase 8 mutations in two children born of consanguineous parents [42]. The percentage of CD4+ T cells in both patients was significantly below normal. Other heterozygous family members did not have a history of immunodeficiency.

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**Box 2. Group II: predominantly antibody deficiencies**

1. Severe reduction in all serum immunoglobulin isotypes with absent B cells
   - A. Bruton's tyrosine kinase (Btk) deficiency
   - B. \(\mu\) Heavy chain deficiency
   - C. I 5 deficiency
   - D. Ig alpha deficiency
   - E. B-cell linker protein (BLNK) deficiency
   - F. Thymoma with immunodeficiency

2. Severe reduction in at least two serum immunoglobulin isotypes with normal or low numbers of B cells
   - A. CVID disorders
   - B. Inducible costimulator (ICOS) deficiency
   - C. CD19 deficiency
   - D. Transmembrane activator and clacium-modulator and cyclophilin ligand interactor deficiency
   - E. BAFF receptor deficiency

3. Severe reduction in serum IgG and IgA with increased IgM and normal numbers of B cells (previously classified as autosomal HIGM syndromes)
   - A. AID deficiency
   - B. UNG deficiency

4. Isotype or light chain deficiencies with normal numbers of B cells
   - A. Immunoglobulin heavy chain deletions
   - B. k Chain deficiency
   - C. Isolated IgG subclass deficiency
   - D. IgA with IgG subclass deficiency
   - E. Selective IgA deficiency

5. Specific antibody deficiency with normal immunoglobulin concentrations and normal numbers of B cells

6. Transient hypogammaglobulinemia of infancy.
Primary

Absent B cells: \( Th^+ Tc^+ B^- NK^+ \)

The absence of B cells can be detected readily in the routine immunophenotyping panel (described previously) by two separate and supporting results: few events are \( CD3^- \) and \( CD19^+ \) and \( CD3^- \) and \( HLA-DR^+ \) bright events are absent (Fig. 9). Mutations in genes that result in arrested B-cell maturation can be separated broadly into (1) X-linked and (2) autosomal recessive.

![Diagram](image)

**Fig. 9.** Routine immunophenotyping results in a patient who had laboratory results and a clinical history consistent with XLA. Patient’s peripheral blood (left column) completely lacks \( CD19^+ \) cells and bright \( HLA-DR^+ CD3^- \) cells consistent with XLA. The absolute lymphocyte count in the 11 month old was 3300 cells/mm³ (within age-associated reference range) and the serum immunoglobulin levels (IgG, IgA, and IgM) all were greater than 2 SDs below normal. Patients who have autosomal forms of agammaglobulinemia can express the identical immunophenotypic abnormalities and without a consistent family history must have sequencing performed to identify the underlying molecular abnormality.
(rarely autosomal dominant) inheritance forms. The majority of patients who have extremely low or absent B cells are men who have a mutation in the gene encoding Btk \[43,44\].

**Measuring Bruton’s tyrosine kinase by flow cytometry**

Abnormal expression of Btk in platelets and monocytes can be assessed by flow cytometry. Peripheral blood labeled with CD14 PE followed by the fixation, permeabilization, and intracellular labeling with a monoclonal antibody to Btk and FITC-conjugated goat antimouse IgG1 identified abnormal Btk protein expression in the monocytes of 40 of 41 patients and cellular mosaicism in 35 of 41 obligate carriers \[45\]. This test provides a fast assessment of Btk expression; however, patients who have missense mutations in the Btk gene can express normal levels of Btk by flow cytometry. Measuring Btk in platelets, Futatani and colleagues observed that Btk was expressed abnormally in 37 out of 45 unrelated families who had X-linked agammaglobulinemia (XLA) and that obligate XLA carriers had normal and abnormal expressing platelets \[46\]. In eight of the families, detection of an abnormality by flow cytometry was not possible because of the normal levels of mutated Btk expression. Patients who had detectable (even very low levels) of Btk protein as assessed flow cytometrically in monocytes presented with significantly higher percentages of B cells (0.7%) compared with patients who had XLA and no detectable Btk protein (0.1% B cells) \[47\]. No relationship was observed between the level of expression of Btk in platelets and the clinical phenotype in the report by Futatani and colleagues \[46\]. Although flow cytometry is useful in detecting patients who have abnormal Btk expression, the absence of the abnormality does not rule out a mutation in the Btk gene and sequencing is highly recommended.

**Autosomally inherited agammaglobulinemia**

In a smaller proportion of patients who have agammaglobulinemia, failure of B-cell maturation is associated with mutations in the genes encoding the B-cell receptor gene, IgM; the surrogate light chain, lambda 5/14.1; the B-cell signaling receptor, Ig alpha; and BLNK \[8\]. These patients experience clinical findings similar to patients who have XLA. A novel form of agammaglobulinemia is inherited in an autosomal dominant fashion (rare for immunodeficiency disease) \[48\] and is caused by mutations in the leucine-rich repeat-containing 8 gene \[49\]. Patient B cells were completely absent in the peripheral blood and the patient cells in the bone marrow expressed the normal and the mutant protein. Surface marker analysis of the B-cell lineage indicated that arrest had occurred at the pre–B-cell stage (slightly earlier than in XLA) \[48\].
Memory B cells in common variable immunodeficiency and X-linked hyper-IgM syndromes

Peripheral blood memory B cells can be identified immunophenotypically by cell surface expression of CD27 [50]. Human peripheral blood contains approximately 35% memory B cells (although this is age related) and the majority are of the nonisotype-switched variety (ie, do not secrete and express immunoglobulins other than IgM and IgD) [51]. The basic flow cytometry procedure used to identify CD27+ memory B cells is illustrated in Fig. 10.

CVID represents a heterogeneous group of diseases with a variety of underlying genetic causes. The identification of the absence of memory B cells in some patients who have CVID [52] has resulted in attempts by to classify patients who have CVID based on the level of this subset [53,54]. Cunningham-Rundles’ laboratory adopted the classification system developed by Warnatz to evaluate a group of 53 patients who had CVID [55]. Patients who had less than 0.4% switched memory B cells (CD19+CD27+IgM−IgD−) were categorized as CVID-group I and patients who had greater than 0.4% switched memory B cells were categorized as CVID-group II. Patients who had the lowest numbers of switched memory B cells had the lowest levels of serum IgG, poor responses to pneumococcal vaccine, and increased rates of autoimmune and granulomatous disease (ie, more severe disease) [55]. There are only a few laboratories in the United States currently performing routine memory B-cell analysis in patients who have CVID (and other patients who have associated immunoglobulin abnormalities) but studies, such as that discussed previously, suggest that this procedure may have a role in better characterizing patients at

![Fig. 10. Immunophenotyping for the measurement of memory B-cell levels. Memory B cells can be detected by the surface expression of CD27 on CD19+ (or CD20+) lymphocytes. In the figure, lymphocytes first are gated using CD45 versus right-angle light scatter and then the percentage of B cells expressing CD27 is measured. B cells in the peripheral blood of the patient shown (right-hand dot plot) contains abnormally low percentages of memory B cell and are consistent with a diagnosis of CVID. Indicated in the right quadrant of each dot plot is the percentage of B cells expressing the memory phenotype (CD27+). (Courtesy of Jack Bleesing, MD, PhD, Cincinnati, OH.)](image-url)
higher risk for CVID and eventually may allow for improved identification of the underlying genetic abnormalities. Patients who have the XHIM are reported to express reduced levels of CD27\(^+\) memory B cells [56].

**Markers of autosomal hyper-IgM (now classified with the predominantly antibody deficiencies)**

The other molecular defects leading to HIGM syndrome include the autosomal genes: AICDA (formerly HIGM2) and UNG. Unlike patients who have the CVID syndrome, B cells from most patients who have the autosomal form of the HIGM syndrome express CD27, implying that the generation of memory B cells in these patients is not necessarily impaired even with an inability to switch immunoglobulin classes [57,58]. In vitro induction of class switch recombination with soluble CD40 ligand and appropriate cytokines (from IgM\(^+\)IgD\(^+\) to other immunoglobulin classes) is impaired in the autosomally inherited forms of HIGM but occurs normally in the XHIM [58].

Mutations in the immunoglobulin heavy chain genes and in the kappa constant gene do not affect circulating B cells numbers and patients usually are asymptomatic. Detection of kappa-chain deficiency is observed easily after surface staining with the appropriate anti–light chain antisera and a B-cell marker.

**Wiskott-Aldrich syndrome and Wiskott-Aldrich syndrome protein**

WAS is an X-linked recessive disease characterized by thrombocytopenia (with small platelets), eczema, and immunodeficiency, both humoral and
cellular [59]. It is associated with mutations in the gene encoding the WAS protein (WASP). Monoclonal antibodies specific for WASP [60,61] have been combined with intracellular flow cytometry staining for the diagnosis of WAS and WAS carriers [61–64], to evaluate mixed chimera status in patients who have WAS post bone marrow transplant [65], and to separate patients who have WAS from those who have thrombocytopenias of unknown cause [64].

*Ataxia telangiectasia and the ataxia-telangiectasia-mutated gene*

The specific combination of immunodeficiency, telangiectasias, and cerebellar ataxia suggests the diagnosis of AT. The disease is inherited in an autosomal recessive fashion and is caused by mutations encoding the gene, ataxia telangiectasia mutated (ATM). Diagnostic confirmation includes testing serum α-fetoprotein (AFP), abnormalities in the routine immunophenotyping assay, and assessment of radiosensitivity [66–68]. Routine flow cytometry confirms the presence of abnormalities in AT; however, the abnormalities are not specific. To date, there are no reports of successfully detecting abnormal expression of the ATM protein by flow cytometry. Heinrich and colleagues [69] published a summary of flow cytometry–based cell cycle testing in a group of 330 patients referred for “exclusion of AT” and were able to ascertain AT negative versus AT positive in 94.2% of the cases tested. Although the investigators concluded that “cell-cycle testing complemented AFP measurements and fulfills the criteria as a rapid and economical screening procedure for the differential diagnosis of juvenile ataxias” [69], I do not think that this particular functional flow–based assay is ready for adoption in a routine clinical setting.

*DiGeorge syndrome*

DiGeorge syndrome is characterized by classical facial features and defects of the heart, parathyroid glands, and thymus [70]. Although DiGeorge syndrome often is grouped with the 22q11.2 deletion syndrome, only approximately 90% of the patients have this anomaly [71]. Defects in the thymus result in variable degrees of immunodeficiency. Those who have below-normal T-cell function (in vitro mitogen-induced proliferative responses) or low T-cell numbers are referred to as having partial DiGeorge, whereas those who have no T-cell responses and no peripheral T cells are referred to as having complete DiGeorge. Although rare, at less than 1% of all patients who have DiGeorge syndrome, complete DiGeorge is a medical emergency and unless patients are treated successfully they die usually before age 1 year. Flow cytometry is useful for assessing the level of immunodeficiency (complete versus partial) and may be associated with increased risk for infection [30]. Significant abnormalities in the TCR V beta repertoire also are observed at baseline [30]. Longitudinal studies of patients who have partial DiGeorge usually show improvement in the immunologic
defects over time [30,72], which may be the result of a disease-associated reduction in the normal rate of T-cell decline observed with aging [73].

**Familial hemophagocytic lymphohistiocytosis**

Hemophagocytic lymphohistiocytosis (HLH) is categorized as familial or secondary. HLH is believed triggered by Epstein-Barr virus, cytomegalovirus, and other viruses, and clinical signs and symptoms include hepatosplenomegaly, liver dysfunction, fever, cytopenias, hyperlipidemia, and increased hemophagocytes in the reticuloendothelial system (especially bone marrow and liver) [74]. Familial forms usually are fatal in infancy unless patients undergo bone marrow transplant, whereas most secondary forms respond to appropriate treatment [75]. Three genes (encoding perforin, Munc 13-D, and syntaxin 11) are known to be associated with the familial form of the disease [76]. Perforin abnormalities present in 20% to 30% of all patients who have HLH [77] can be detected by the intracellular staining of NK cells. Ueda and colleagues [77] examined perforin protein levels in the mononuclear cells from 19 patients who had FHL, and observed abnormal perforin expression levels (percent positive and mean fluorescent intensity) in cytotoxic T cells (CD8+) and NK cells (CD16+/CD56+) in six of the samples. Perforin protein is reported to be “uniformly deficient” in patients who have FHL and perforin.

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**Box 4. Group IV: diseases of immunodysregulation**

1. Immunodeficiency with hypopigmentation  
   A. Chédiak-Higashi syndrome  
   B. Griscelli syndrome type 2  
2. Familial hemophagocytic lymphohistiocytosis (FHL) syndromes  
   A. Perforin deficiency  
   B. Munc 13-D deficiency  
   C. Syntaxin 11 deficiency  
3. X-linked lymphoproliferative syndrome  
4. Syndromes with autoimmunity  
   A. Autoimmune lymphoproliferative syndrome (ALPS)  
   i. CD95 (Fas) defects, ALPS type 1a  
   ii. CD95L (Fas ligand) defects, ALPS type 1b  
   iii. Caspase 10 defects, ALPS type 2a  
   iv. Caspase 8 defects, ALPS type 2b  
   B. Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)  
   C. Immunodysregulation, polyendocrinopathy, and enteropathy (X-linked) (IPEX)
gene mutations [77]; however, one must be cautious of the possibility that a functional mutation could be present even though expression levels as assessed by flow cytometry are normal.

The autoimmune lymphoproliferative syndrome

The molecular basis of this group of chronic nonmalignant lymphoproliferative disorders first was characterized in 1995 as caused by gene encoding Fas [78,79]. Although the majority of patients who have ALPS have mutations in the gene encoding Fas, it is now known that other genes, including Fas ligand, caspase 8, and caspase 10 (all involved in Fas-mediated apoptosis), also lead to ALPS [80]. Impaired programmed cell death results in the accumulation of lymphocyte and causes the clinical phenotype of lymphadenopathy, hypersplenism autoimmune cytopenias, and an increased risk for lymphoma. ALPS diagnosis can be substantiated strongly by the flow cytometric assessment of Fas-induced apoptosis using a variety of methods, including annexin cell surface labeling, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (so-called “TUNEL” assay), hypodiploid nuclear DNA content, and propidium dye exclusion [80]. Flow cytometry can been used to screen patients suspected of having ALPS by measuring for increases in alpha/beta TCR-positive, and CD4/CD8 double-negative T cells [81]. My colleagues and I have developed a panel of antibodies (TCR alpha/beta-FITC, CD4-PE, CD3-PerCP, CD8-APC) (BD Biosciences, San Jose, California) to measure this subset in patients suspected of having ALPS. The procedure for identifying and measuring this subset is illustrated in Fig. 11. Normal ranges for this subset are reported from less than 1% of T cells [81] up to less than 2.6% [82]. The flow cytometric assessment of double-negative T cells is an easy screening test and an increased in the percentage double-negative T cells with the appropriate clinical signs is suggestive of a diagnosis of ALPS.

Autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy

APECED, also known as autoimmune polyglandular syndrome type 1, is an autosomal recessive monogenetic disease characterized (as the name suggests) by endocrine organ-specific autoimmunity and candidiasis [1] although the phenotype is very heterogeneous [83]. The gene identified as associated with APECED is known as autoimmune regulator (AIRE) [84,85]. Measuring the expression levels of AIRE in cells from patients who have suspected APECED by flow cytometry has not been reported. Recent descriptions, however, of the positive expression of AIRE in CD14+ monocytes and differentiated dendritic cells (but not CD4+ T cells and PMNs) by immunocytochemistry suggest that is may be possible to assess AIRE expression by flow cytometry as an aid in the diagnosis of APECED [86].
Immunodisregulation, polyendocrinopathy, and enteropathy (X-linked)

The three most common clinical features of this X-linked recessive autoimmune disorder are early-onset, insulin-dependent diabetes, severe watery diarrhea, and dermatitis with high level of serum IgE [1]. First described in 1982 [87], IPEX now is known to be caused by mutations in the forkhead box P3 (FoxP3) gene located on the X chromosome. In addition to the autoimmune manifestations, patients often succumb to severe infections [83]. Flow cytometry is used to screen patients suspected of having IPEX by the intracellular staining for FOXP3 in CD4+CD25+ T cells. Most patients who have IPEX lack FoxP3-positive cells; however, there is the possibility that the detection of FoxP3 does not necessarily rule out a mutant FoxP3 gene.

Fig. 11. Flow cytometric procedure and gating strategy for the enumeration of CD4 and CD8 double-negative, alpha/beta TCR-positive T cells (CD3+) in patients suspected of a diagnosis of ALPS. First, T cells are identified on a dot plot of CD3 versus right-angle light scatter. A region is drawn around the T-cell cluster (R1) and the number of T-cell events noted. This region then is analyzed in a dot plot of CD4 versus CD8 and a region (R2) is drawn to include the CD4+ and CD8− events. Events included in regions 1 and 2 (ie, CD3+CD4+CD8−) then are analyzed for the number of events that are positive for the alpha/beta form of the TCR. The number of CD3+CD4+CD8− TCR alpha/beta+ events is divided by the number of T cells (27,036 in the upper-left dot plot region 1) to obtain the percentage of CD3+CD4+CD8− TCR alpha/beta+ cells. Patients who have a diagnosis of ALPS can have percentages of above the normal range.
Chronic granulomatous disease: gp91phox, p22phox, p47phox, and p67phox

CGD is a disease affecting the superoxide generation and microbial killing in phagocytes. The defect is the result of abnormal reduced nicotinamide-adenine dinucleotide phosphate oxidase activity as a result of mutations in one of four known subunit genes. CGD is the most common phagocyte defect, affecting approximately 1 in 200,000 persons [88]. The most common form of CGD is caused by mutations to the X-linked gene, gp91phox.
(70% of CGD cases); the remainder of the cases occur as a result of mutations in autosomally encoded genes: p22phox (the other component of membrane flavocytochrome b558), p47phox, and p67phox [89].

Monoclonal antibodies to the cytochrome b558 complex have been developed [90–92] and combined with flow cytometry to diagnose abnormal membrane expression in patients who have CGD [93]. Currently, the use of monoclonal antibodies in the diagnosis of CDG is relegated largely to highly specialized clinical or research laboratories using Western blot methodology to confirm the mutated gene products. Screening for CGD is performed more commonly by flow cytometry using quicker and less expensive functional flow cytometry–based oxidative burst screening assays.

The oxidative burst assay

The principle of the flow cytometry–based procedure is that a nonfluorescent reactive oxygen intermediate-sensitive dye is loaded into granulocytes and after an in vitro induction of the oxidative burst becomes brightly fluorescent. Patients who have CGD are unable to generate an oxidative burst and, therefore, the dye remains nonfluorescent. The use of a flow cytometry–based procedure using dichlorofluorescein diacetate for the diagnosis of CGD was reported first by Bass and coworkers in 1983 [94]. The discovery of a more sensitive dye led [95] to the development of new flow cytometry–based assays for the diagnosis of CGD; however, the tests still were performed on samples that had been processed before dye loading and stimulation [96]. My colleagues and I developed an oxidative burst assay using the uncharged nonfluorescent dihydrorhodamine 123, which could be added directly to whole blood, followed by stimulation, lysis, and fixation, which required much less blood and time than previously published methods [97]. Briefly, dye is added directly to diluted whole blood and incubated in a shaking water bath at 37°C for 15 minutes followed by stimulation with phorbol 12-myristate 13-acetate (PMA) for an additional 15 minutes. The red blood cells then are lysed with an ammonium chloride solution and the remaining cells fixed in 1% paraformaldehyde and run immediately on the flow cytometer. Results are expressed as a normal oxidative index (NOI), which is a ratio of the fluorescence in stimulated cells to the fluorescence observed in unstimulated cells. The normal range for NOI in my laboratory is greater than 30 (usually over 100), whereas most patients diagnosed with CGD usually generate NOI results close to 1 (ie, very little change in fluorescence after in vitro stimulation). Rarely, patients who have either the X-linked form or the autosomal form of the disease produce NOI results approaching the normal range (personal experience). It has been observed that patients who have the autosomal form of CGD (p47phox) have significantly higher NOI than patients who have CGD with X-linked CGD (gp91phox) and that the fluorescence levels are more variable (increased CV) than the patients who have X-linked CGD and controls [98].
Biologic mothers of patients who have an abnormal NOI should be tested for carrier status. Those mothers whose granulocytes generate two clearly different levels of fluorescence (one normal $>30$ and one abnormal $<30$) confirm the diagnosis of X-linked CGD in the child and the status as a carrier of the X-linked mutation. My colleagues and I have diagnosed X-linked CGD carriers successfully with as few as 15% normal granulocytes. Parents of patients who have CGD with the autosomal forms of the disease usually generate oxidative burst assay results consistent with normal (Maurice O’Gorman, PhD, unpublished data, 2007 personal experience). The assay can be very sensitive. Using a procedure slightly different from ours, Vowells and colleagues [99] reported being able to detect 0.1% normal granulocytes consistently when mixed with cells from a patient who had CGD. The test can be performed reliably on sodium heparin anticoagulated blood samples up to 72 hours old [100], although this needs to be validated in each individual laboratory. Recently, a whole blood procedure was described that did not involve lysis or wash steps; however, the results of this generated a fourfold lower “activity index” (equivalent to the NOI) than the lyse wash method [101]. It does not seem as though this new method will be used widely as a diagnostic test. Fig. 12 provides an illustration of the simple gating strategy and the results obtained from a normal control, a patient who had X-linked CGD, and the mother (an X-linked carrier) of the patients who had X-linked CGD.

**Leukocyte adhesion deficiency type 1, CD18, CD11a, CD11b, and CD11c cell surface expression abnormalities**

LAD-1 is a rare disorder characterized clinically by defective wound healing, infections by gram-negative bacteria without pus, delayed umbilical cord separation, gingivitis, and periodontitis. The genetic cause of the disease results from mutations in the gene-encoding beta chain (CD18) of the $\beta_2$ class of leukocyte integrins. Abnormal CD18 expression results in its inability to carry the $\alpha$ chain complexes (CD11a, CD11b, and CD11c) to the cell surface. In 1992, a patient who had a clinical history and laboratory findings consistent with LAD-1 presented to our emergency room and proved to have cell surface abnormalities consistent with LAD-1. With their informed consent, we were able to develop a whole blood flow cytometry–based diagnostic test [102]. Briefly, an optimized concentration of phorbol myristate acetate is added to whole blood for 15 minutes followed by the staining of the whole blood with a monoclonal antibody directed against CD11b (CD11b-PE, BD Biosciences). EDTA anticoagulated whole blood labeled with an isotype-matched nonleukocyte-specific monoclonal antibody is used to measure the baseline level of fluorescence of the granulocyte cluster (using only forward- versus right-angle light scatter parameters) as compared with CD11b expression on resting and activated granulocytes. Normal ranges were established using whole blood from 30 nondiseased
control subjects. Patients who have LAD-1 express CD11b significantly below normal (less than 5% of normal levels) on resting and stimulated granulocytes. Patients who have severe LAD-1 do not survive infancy and express no detectable CD11a, CD11b, or CD11c protein on the cell surface,
whereas patients who have the moderate form of LAD-1 express 1% to 10% of the level of normal and often survive into adulthood [103].

*Interferon-γ receptor 1, interferon-γ receptor 2, interleukin-12, and interleukin-12 and interleukin-23 receptor-beta 1 chain: interferon-γ pathway defects: increased susceptibility to poorly pathogenic mycobacteria, salmonella, and other intracellular infectious pathogens*

Unusually severe infections with intracellular organisms, including bacille Calmette-Guérin, nontuberculous mycobacteria (*Mycobacterium avium, M. fortuitum*, and *M. chelonae*), and salmonella, have led to the identification of mutations in several genes involved in the IFN-γ pathway (reviewed in Refs. [104,105]). The IFN-γ pathway involves IFN-γ receptors (IFN-γR1 and IFN-γR2), IL-12 (IL-12p40), and an IL-12 receptor (IL-12Rβ1). IL-12 stimulates T cells and NK cells to synthesize and secrete INF-γ, which in turn activates macrophage and further stimulates cytolytic CD4+ T cells and effector CD8+ T cells, which then kill infected macrophage [106]. Abnormalities in IL-12 and IFN-γ receptors are reported and can be detected as increased and decreased expression levels by flow cytometry. In a dominant form of the IFN-γ receptor mutation, the expression of the receptor actually is increased on the cell surface [104,105,107]. The lack of specificity in associating surface receptor expression levels to specific genetic mutations has led to the development of functional flow cytometry assays. The latter are based on the ability to detect phosphorylated versus nonphosphorylated kinase substrate components of the IFN-γ pathway after in vitro stimulation with IFN-γ [104].

Lack of STAT1 phosphorylation (detected flow cytometrically) after in vitro INF-γ stimulation indicates abnormal IFN-γ receptor molecules (IFN-γR1 or IFN-γR2) or abnormalities in STAT1 itself, whereas normal in vitro STAT1 phosphorylation response indicates abnormalities in the

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Fig. 12. Results of our flow cytometry–based oxidative burst assay for the diagnosis of CGD in a family with an unusual case of X-linked CGD. In the top dot plot of the left column, granulocytes are gated based on their inherent characteristic light scatter properties. In all of the remaining histograms, the NOI results are expressed as the median fluorescence channel of the in vitro activated granulocytes with dye (dark peaks in the histograms) divided by the median fluorescence channel of the granulocytes, which have dye but have not been activated (light peaks in the histograms). The histogram at the top in the right column from a healthy control (run with every patient) is consistent with normal (NOI > 30). The middle histograms in the left and right columns are from brothers suspected of having CGD. The results were difficult to interpret as they were very close to our normal cutoff of 30 (NOI on CDG patient = 30, NOI on brother = 22). Repeats on the same samples generated similar results. A sample from the mother was obtained and clearly showed two peaks, one normal (NOI = 187) and one abnormal (NOI = 30). The results on the mother clarified the probable patient diagnosis and were reported as consistent with X-linked CGD for the two sons and consistent with an X-linked carrier of CGD for the mother (samples were sent for sequencing of the gp91phox gene to confirm the diagnoses; however, the results are not yet available).
IL-12 gene or the IL-12 receptor genes. The diagnosis of patients who have these disorders and the determination of the underlying genetic mutation is important because (1) the prognosis differs with the different forms of genetic lesions and (2) there may be implications for the design of optimal therapeutic interventions [105,108]. The flow cytometry assays developed help direct target gene sequencing but are performed only in specialized facilities. Fig. 13 illustrates the results of the STAT1 phosphorylation assay in a patient who had an autosomal dominant INF-γR1 mutation at nucleotide 818 of the INF-γR1 gene, which resulted in a truncated receptor with impaired signaling and recycling [105]. Abnormal signaling results in reduced IFN-γ–induced STAT1 phosphorylation, and impaired recycling results in increased surface expression of IFN-γR1 (see Fig. 13).

Fig. 13. Flow cytometric assessment of the effects of different mutations in the IFN-γR1 gene on the expression levels of IFN-γR1 on the surface of monocytes. Illustrated are examples of INF-γR1 levels on the peripheral blood monocytes obtained from a normal control (bottom left), an autosomal recessive mutation (bottom middle), and an autosomal dominant mutation (bottom right). The histogram in the middle illustrates the below-normal levels of INF-γR1 (dark peak) overlayed on the isotype control (light peak) in a patient who had a mutation in exon III (211-2) (A→G) of the INF-γR1 gene. The histogram on the right is an example of the increased cell surface expression of IFN-γR1 due to a dominantly inherited mutation (818 del 4). This class of mutation results in the deletion of the recycling domain, the JAK1 and the STAT1 motifs. Mutations at this site result in a frame shift that in turn leads to a premature stop (truncated mRNA) and the early termination of the protein [104]. The abnormal protein has increased expression levels on the cell surface (due to lack of the recycling domain), is nonnonsignaling and competes with the wild-type receptors for IFN-γ [104]. (Courtesy of Gulbu Uzel, MD, Chicago, IL)
The PID that are classified as Table VI (defect in innate immunity), Table VII (autoinflammatory disorders) and Table VIII (complement deficiencies) are not addressed in this article. It is conceivable, however, that flow cytometry applications may be developed to investigate abnormalities in these PID in the future.

**Summary**

Flow cytometry has been and continues to be an invaluable tool for the diagnosis and monitoring of patients suspected of having PID.

**References**


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