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# Laboratory Diagnosis of Primary Immunodeficiencies

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**Abstract** Primary immune deficiency disorders represent a highly heterogeneous group of disorders with an increased propensity to infections and other immune complications. A careful history to delineate the pattern of infectious organisms and other complications is important to guide the workup of these patients, but a focused laboratory evaluation is essential to the diagnosis of an underlying primary immunodeficiency. Initial workup of suspected immune deficiencies should include complete blood counts and serologic tests of immunoglobulin levels, vaccine titers, and complement levels, but these tests are often insufficient to make a diagnosis. Recent advancements in the understanding of the immune system have led to the development of novel immunologic assays to aid in the diagnosis of these disorders. Classically utilized to enumerate lymphocyte subsets, flow cytometric-based assays are increasingly utilized to test immune cell function (e.g., neutrophil oxidative burst, NK cytotoxicity), intracellular cytokine production (e.g., TH17 production), cellular signaling pathways (e.g., phosphor-STAT analysis), and protein expression (e.g., BTK, Foxp3). Genetic testing has similarly expanded greatly as more primary immune deficiencies are defined, and the use of mass sequencing technologies is leading to the identification of novel disorders. In order to utilize these complex assays in clinical care, one must have a firm understanding of the immunologic assay, how the results are interpreted, pitfalls in the assays, and how the test affects treatment decisions. This article will provide a systematic approach of the evaluation of a suspected primary immunodeficiency, as well as provide a comprehensive list of testing options and their results in the context of various disease processes.

**Keywords** Primary immunodeficiency · Diagnosis · Laboratory assessment · Flow cytometry

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## Introduction

Over the past 25 years, extensive research and technological advancements have furthered the scientific understandings of the immune system. Clinical immunologists have been able to capitalize on these scientific advancements by translating basic science findings to patients, thus providing improvements in the diagnosis and treatment of primary immunodeficiencies. The classification of primary immunodeficiencies has expanded as a result of this increased knowledge, and there are currently greater than 180 known primary immunodeficiencies [1]. Physicians are now able to diagnose immune disorders much earlier, provide more effective and targeted treatment, reduce patient morbidity/mortality, and enhance their patient's quality of life. This chapter will focus on the laboratory tests and interpretations that should ideally be considered in various primary immunodeficiencies.

### Disorders of Humoral Immunity

Approximately 50–60 % of all identified primary immunodeficiencies are caused by defects in antibody production [2]. Individuals with these disorders typically develop recurrent

sinopulmonary infections related to encapsulated bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. These patients also are known to have an increased risk of developing infectious diarrhea, typically due to *Giardia*, rotavirus, enterovirus, *Campylobacter*, *Salmonella*, *or Shigella* [3]. Humoral defects have also been associated with an increased incidence of developing autoimmunity, enteropathy, granulomatous disease, and lymphocytic infiltrate of the lung [4, 5].

The most important initial screening tests when a humoral immunodeficiency is suspected are evaluations of quantitative immunoglobulins (IgG, IgA, IgM, and IgE) (Table 1). It is critically important that the results of these tests be compared to age-adjusted normal values, particularly in children. In general, hypogammaglobulinemia is defined when the value is less than two standard deviations below the age-adjusted normal. Additionally, agammaglobulinemia is defined by an IgG of less than 100 mg/dL. If either of these findings is found, then further immunologic workup should be pursued.

Since antibody specificity is as important as immunoglobulin levels, assessment of specific antibody titers is also critical in the evaluation of a suspected humoral immunodeficiency. This assessment can be done by evaluating serum titers to common vaccinations, such as the tetanus, diphtheria, pneumococcus, and *Haemophilus influenzae* type b (Hib). Tetanus and diphtheria are protein-based vaccines which generate strong, long lasting immunity. On the other hand, polysaccharide-based vaccines (i.e., pneumococcus) generate a weaker immunologic memory response [6]. If any serum vaccination titers are below normal, revaccination and assessment of titers 4–6 weeks later is warranted. There is controversy regarding "normal" response to vaccination, particularly to polysaccharide vaccine. However, some groups define the following criteria as an adequate vaccination response: (1) a measured protective titer per lab normals; (2) a fourfold increase in post-vaccination titer level compared to pre-vaccination titer; and (3) a measured response to >50 % of polysaccharide serotypes tested from ages 2–5 years old or a response of >70 % in patients greater than 5 years of age [7]. These criteria have not been validated among all immunodeficiencies, but they did demonstrate validity with 73 % sensitivity and 57 % specificity in a cohort of children infected with the human immunodeficiency virus [8].

A significant dilemma when attempting to evaluate the humoral immune system occurs in individuals receiving immunoglobulin replacement therapy since immunoglobulin preparations contain detectable titers to common vaccines. Currently, the options for evaluation in these patients are limited. However, in select medical centers, patients are able to be vaccinated with a neoantigen, bacteriophage Phi X174, and antibody responses evaluated [9]. Research is also being done regarding the use of the rabies vaccine and the *Salmonella typhi* Vi vaccine for this purpose [10].

If initial screening of quantitative antibody levels or specific antibody production yields concerning results, additional testing can be done such as flow cytometry to evaluate lymphocyte numbers and maturation, or genetic testing for mutations to cause humoral defects. Finally, there are other testing modalities available in select medical centers which are able to evaluate for B cell signaling defects and problems with immunoglobulin biosynthesis [11].

**Table 1** Algorithm to evaluate of a suspected humoral immune defect

| | |
|---|---|
| Screening evaluation | • CBC with differential |
| | • Quantitative immunoglobulins |
| | ○ (IgG, IgA, IgM, IgE) |
| | • Baseline and post-immunization titers |
| | ○ Protein and polysaccharide antigens (i.e., Pneumococcal, Diphtheria) |
| | • CH50/AH50 |
| | • Chemistry panel |
| | • Urinalysis |
| Advanced testing | • B-cell maturation assessment by flow cytometry |
| | • Assessment of other immunizations |
| | • B-cell signaling assays |
| | • TREC/KREC analysis |
| | • Advanced flow cytometry studies |
| | ○ i.e., BTK presence, pH2AX immunofluorescence |
| | • Genetic mutational analysis |
| | ○ i.e., BTK, TACI, ATM mutations (Known disease causing mutations) |

*CBC* complete blood count; *IgG* immunoglobulin G; *IgA* immunoglobulin A; *IgM* immunoglobulin M; *IgE* immunoglobulin E; *CH50* total hemolytic complement activity; *AH50* alternative pathway hemolytic activity; *TREC* T-cell receptor excision circles; *KREC* kappa rearrangement excision circles; *BTK* Bruton's tyrosine kinase; *pH2AX* phosphorylated H2A histone family, member X; *TACI* transmembrane activator and calcium modulator and cyclophilin ligand interactor; *ATM* ataxia-telangiectasia mutated

### *Common Variable Immunodeficiency*

One of the most common diseases that results in a significant humoral deficit is common variable immunodeficiency (CVID). The diagnosis of CVID requires a low IgG with a low IgA and/or IgM, as well as defective antibody response to vaccination [12]. Significant sources of morbidity in addition to infection in these patients include the development of bronchiectasis, enteropathy, polyclonal lymphocytic infiltration, granulomatous disease, and autoimmunity [5]. Flow cytometry can be used to evaluate the maturational state of B lymphocytes based upon the expression of IgM, IgD, CD27, and CD38. Mature, naïve B lymphocytes express IgM and IgD, but do not express CD27 and CD38. Upon

antigen activation, B lymphocytes upregulate the activation/memory marker CD27 and become memory B cells. The memory B-cell compartment can be further divided between those expressing surface IgD (unswitched memory B lymphocytes) and those not expressing surface IgD (switched memory B lymphocytes) (Fig. 1a). Switched memory B cells produce IgG and IgA. CD38 expression can also be used to evaluate for antibody-secreting cells and plasma cells.

B-cell immunophenotyping of CVID patients demonstrates low numbers of switched memory B cells ($CD27^+$, $IgM^-$, $IgD^-$) approximately 50–75 % of the time[13] (Fig. 1b). The memory T-cell compartment can also demonstrate abnormalities, with a reduced $CD4^+/CD8^+$ ratio and diminished percentage of $CD4^+$ T cells expressing CD45RA [14]. There have been multiple classification systems used to define CVID; however, the most recent system is based upon data from a large collaborative patient cohort (EUROClass) that uses a combination of switch memory B-cell populations and other markers to classify CVID. First published in 2008, the EUROClass nomenclature demonstrated that patients with severely reduced (<2 %) switched memory B cells and >10 % $CD21^-/CD38^-$ B cells were noted to be associated with splenomegaly and the development of granulomatous disease [15]. The presence of $CD21^-$ B cells is seen in patients who are more likely to develop autoimmunity [16]. Finally, lymphoid malignancy in a patient with CVID has also shown a positive correlation with IgM level at diagnosis [5].

Autosomal dominant mutations in tumor necrosis factor receptor superfamily 13 B (*TNFRSF13B*), otherwise known as transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), account for approximately 10 % of individuals diagnosed with CVID [17]. TACI-associated CVID is linked to an increased incidence of splenomegaly, tonsillar hypertrophy, and autoimmune thyroiditis [18]. TACI itself is expressed on memory B cells and can be detected by flow cytometry; however, few TACI-deficient patients present with absent or severely reduced TACI surface expression [19]. Inducible T-cell co-stimulator (ICOS), CD19, and tumor necrosis factor receptor superfamily member 13C (TNFRSF13C, also known as BAFFR) deficiency are autosomal recessive disorders and each account for less than 1 % of clinical CVID cases [20-22]. A previously described cohort of patients with ICOS-associated CVID developed antibody deficiency, nodular lymphoid hyperplasia, autoimmunity, and an increased association with malignancy [23]. Flow cytometry can be used to demonstrate reduced expression of protein markers TACI, CD19, and BAFFR, but this should be paired with genetic evaluation for these defects [18, 21, 22].

### *X-Linked Agammaglobulinemia*

Patients diagnosed with congenital agammaglobulinemia classically develop sinopulmonary infections around the age of 4–6 months as the maternally transferred IgG declines. The most common cause of congenital agammaglobulinemia is X-linked agammaglobulinemia (XLA) due to a genetic defect in the Bruton's Tyrosine Kinase (*BTK*) gene. This disorder accounts for approximately 85 % of agammaglobulinemia, with the remaining 15 % due to autosomal recessive mutations [24]. BTK is a protein tyrosine kinase required for B-lymphocyte development, and patients typically have a block in B-cell development at the pre-B-cell stage. Over 50 % of XLA patients present prior to one year of age, with more than 90 % being diagnosed before 5 years old [25]. Laboratory evaluation will yield low levels of IgG, IgA, and IgM; and absent immunization titers [26, 27]. Lymphocyte subset analysis will demonstrate a low/absent circulating B cell population with normal T-cell counts [28]. Neutropenia can occur in up to 25 % of XLA patients [29]. By using flow cytometry, one can evaluate for intracellular expression of BTK in monocytes since B cells are typically absent (Fig. 2). The inability to identify BTK protein is a strong diagnostic indicator of XLA [30]. It should be noted that this modality of analysis does not pick up all mutations, only those that affect protein stability. For this reason, it is recommended to perform *BTK* sequence

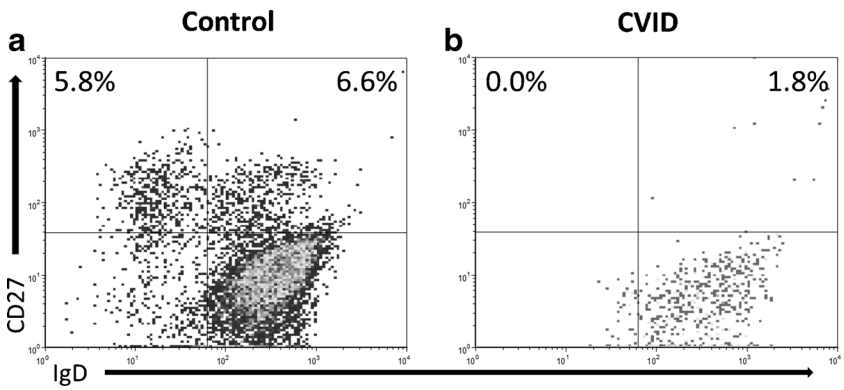


**Fig. 1** Abnormal B cell maturation in common variable immunodeficiency. Whole blood was analyzed with antibodies to CD19, IgD, and CD27. **a** Normal distribution of naïve ($IgD^+$ $CD27^-$), unswitched memory ($IgD^+$ $CD27^+$), and switch memory ($IgD^-$ $CD27^+$) B cells ($CD19^+$) in a healthy subject. **b** Decreased distribution of switch memory B cells seen in cases of CVID. *CVID* common variable immunodeficiency, *IgD* immunoglobulin D

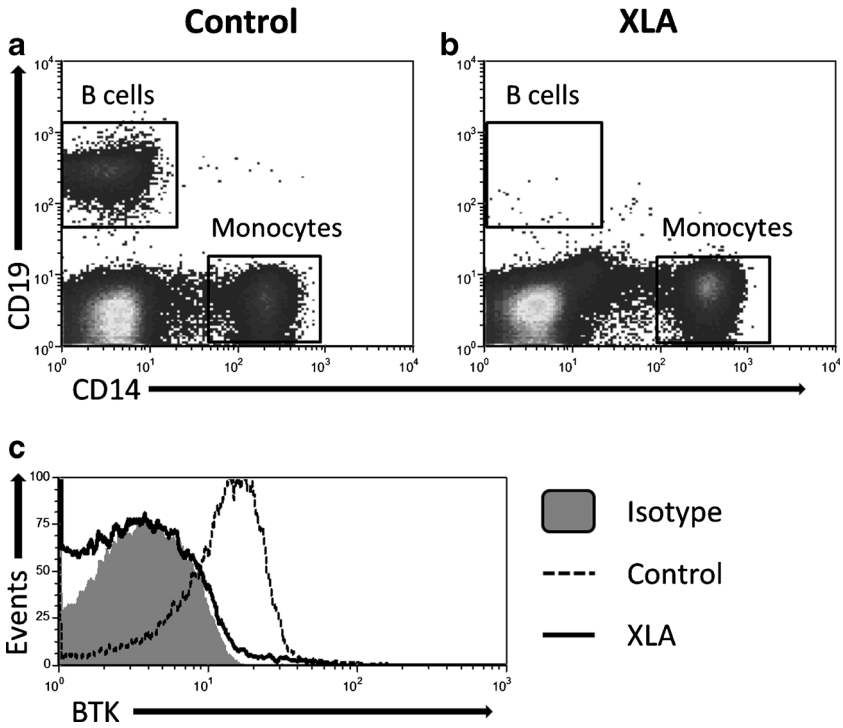


**Fig. 2** BTK expression in monocytes for evaluation of X-linked agammaglobulinemia. Whole blood was analyzed with antibodies to CD14 (monocyte marker), CD19, and BTK protein. **a** Detection of monocytes and B cells in a control specimen. **b** Undetectable B-cell population in a patient with XLA. **c** Measured BTK expression in monocytes of control versus XLA patient. *XLA* X-linked agammaglobulinemia, *BTK* Bruton's tyrosine kinase

analysis in patients with a clinical picture consistent with XLA. Some patients with *BTK* mutations have been misdiagnosed as CVID patients who may present later with low B-cell counts and low-level antibody production [31, 32]. For this reason, it is important to recognize and evaluate for a *BTK* mutation in cases which may clinically correlate with CVID [24].

Congenital agammaglobulinemia is among disorders that may be detected prior to the development of infectious episodes using population-based screening programs by quantification of kappa-deleting recombination excision circles (KRECs). Similar to how the T-cell replication excision circle (TREC) screening has helped lead to the earlier diagnosis of disorders with severe T cell lymphopenia (i.e., severe combined immunodeficiency), analysis of KRECs at birth has the potential to detect any congenital disorders affecting B-cell development. KRECs are episomal DNA fragments generated during the rearrangement process of the kappa light-chain genes during B-cell development [33]. By using polymerase chain reaction (PCR) of newborn blood spots to quantitate these non-replicative DNA elements, one is able to effectively screen for disorders which disrupt B-cell development, such as with X-linked agammaglobulinemia, ataxia-telangiectasia, and Nijmegen breakage syndrome [34].

### *Ataxia-Telangiectasia*

Ataxia-telangiectasia (AT) typically presents with progressive cerebellar ataxia, cutaneous or conjunctival telangiectasias, and recurrent infections. These patients typically have a markedly decreased serum immunoglobulins and poor cell-mediated responses [35]. Importantly, these patients demonstrate sensitivity to γ-radiation which normally disrupts cell cycle checkpoints and induces DNA repair mechanisms [36]. However, radiation sensitivity testing has a long turnaround time, is not widely available, and abnormal results are not specific for AT [37]. Flow cytometry can provide some ancillary data, as it has previously been shown that a large portion of these patients have a relative increase in the γδ T-cell population and an inappropriate expansion of T-cell clones leading to a low number of $CD4^+/CD45RA^+$ T lymphocytes in these patients [38, 39]. KREC analysis by RT-PCR has been shown to detect AT. These patients may be identified by alterations of the ataxia-telangiectasia mutated (ATM) protein or phosphorylated histone H2AX by flow cytometry [40]. In humans, approximately 10–15 % of histone H2A is made up of H2AX. After exposure to ionizing radiation, DNA repair mechanisms induce phosphorylation of H2AX to γ-H2AX. Due to *ATM* gene defects in patients with AT, they do not recognize DNA defects and thus do not phosphorylate H2AX. Using flow cytometry to quantify γ-H2AX in T-cell lines, lymphoblastoid cell lines, and peripheral blood mononuclear cells, previous studies have shown that there is virtually 100 % sensitivity and specificity of detecting patients with AT versus healthy controls [40].

## Cellular and Combined Immune Defects

T lymphocytes are a central component of the adaptive immune system. Defects that affect T-cell function or number

prevent proper B-cell function which can lead to defective antibody production and susceptibility to infectious agents that are controlled by antibodies [6]. In addition, T cells are essential for cell-mediated immunity that is critical to the control of intracellular pathogens, viruses, and opportunistic infections. $CD4^+$ T-cell activation of phagocytes by Th1-cytokines or CD40L (see below) enables them to clear intracellular pathogens, fungi, and protozoa. $CD8^+$ T cells are essential to control viral infections. Patients with T-cell defects experience serious and frequent infections of the skin, respiratory system, or gastrointestinal system. These infections may also be more difficult to treat with traditional therapies and include opportunistic pathogens that are non-virulent in an immune-competent host [41].

When concerned for an underlying cellular immune defect, one the most common and most overlooked pieces of laboratory data is the absolute lymphocyte count (ALC) (Table 2). The ALC should be compared to age-adjusted normals since infants have a much higher lymphocyte count than adults [42]. As a general guideline, an infant with an ALC of less than $3{,}000/mm^3$ should prompt evaluation for a possible immune defect. If presented with a patient with a decreased lymphocyte count, possible infection due to HIV must be evaluated as part of an immunologic workup. It is important to evaluate the HIV viral load by PCR, as affected patients will not be able to generate an anti-HIV-antibody response.

The second tier evaluation of suspected T cell or combined T/B cell defects is lymphocyte enumeration by flow cytometry. Flow cytometry allows for the discrimination of CD4, CD8, natural killer (NK), and B cell numbers. This is important as one cannot adequately judge cell numbers by complete blood cell count and differential alone since selective deficiency of a lymphocyte subset can be missed. Immunophenotyping is especially useful in the diagnosis of severe combined immunodeficiency, as the pattern of missing cell types helps to delineate the immunologic defect present. Although low T-cell numbers are typically observed in the majority of defects in T-cell development, this may be masked due to transplacental transfer of maternal T lymphocytes. Therefore, analysis of markers of cell activation should be analyzed since the transferred T cells will activate and expand in the infant. Typically, maternal T cells will display a memory ($CD45RO^+$) phenotype, whereas a healthy infect should have predominantly naïve $CD45RA^+$ T cells [43]. Also, non-filtered/non-irradiated blood transfusions can lead to T-cell engraftment in patients with severe combined immunodeficiency which can result in a potentially fatal graft-versus-host disease.

Another commonly available test that evaluates for T-cell functional defect is cutaneous delayed-type hypersensitivity (DTH) testing. This is a test which measures a cellular-mediated memory response to a previously seen antigen [44]. Typically, at least three different antigens are used in DTH testing, namely purified protein derivative (PPD), *Candida albicans*, and mumps. These antigens are intradermally injected, then evaluated 48–72 h later for cutaneous induration greater than 2 mm. There are some caveats when using this modality of testing which should be taken into account. First, DTH testing requires that there has been previous exposure to the antigen prior to testing [44]. Second, it is not recommended to perform DTH testing on children less than 12 months of age as they are frequently unresponsive due to immunologic maturity [32]. Third, various infections and medications can result in one's DTH results to be falsely negative [45]. Finally, a positive test to some antigens does not ensure normal cellular immunity to all antigens [32].

Lymphocyte mitogen assays are important in the evaluation of patients with presumed cellular defects. Lymphocytes are stimulated with T-cell mitogens for several days, and then added radiolabeled thymidine is incorporated into the DNA of proliferating cells and quantified. The five most common mitogens used in the laboratory include the following: phytohemagglutinin (PHA), concanavalin (ConA), anti-CD3 antibodies, pokeweed (PWM), and *Escherichia coli* lipopolysaccharide (LPS). PHA, ConA, and anti-CD3 antibodies induce a response in T cells, whereas LPS only stimulates B cells. PWM has the ability to stimulate both T cells and B cells [6]. Lymphocyte responses to specific antigens (i.e., tetanus) can also be performed.

Recent implementation of newborn screening via quantification of TRECs has helped identify severe T-cell lymphopenia in infancy, typically prior to the individual's first infection [46-48]. This allows for earlier diagnosis and treatment, reducing morbidity and mortality. TRECs are circular, non-replicating pieces of DNA which are excised during T-cell receptor rearrangement and are a surrogate marker of naïve T cells. Although this is typically used for newborn screening, patients with T-cell defects can also have low TRECs at any age [34].

**Table 2** Algorithm to evaluate a suspected cellular immune defect

| | |
|---|---|
| Screening Evaluation | • CBC with differential |
| | • Immunophenotyping |
| | ○ T-, B-, NK-cell counts and $CD45RA/RO^+$ status |
| Advanced Testing | • Functional testing |
| (*If pertinent, based upon previous testing results or high clinical suspicion*) | ○ DTH testing |
| | ○ Mitogen stimulation |
| | ○ Cytotoxicity assay |
| | • TREC assay |
| | • Genetic evaluation |
| | • Advanced flow studies |
| | ○ i.e., TH17, CD40L, WASp, etc. |

*CBC* complete blood count, *DTH* delayed-type hypersensitivity, *TREC* T-cell receptor excision circles, *CD40L* CD40 ligand, *WASp* Wiskott-Aldrich protein

A recent area of research regarding evaluation of primary immunodeficiencies is T-cell repertoire analysis [49, 50]. Analysis of T-cell repertoire by CDR3 sequence analysis or spectratype analysis can aid in the evaluation of T-cell lymphopenia and give data regarding the T-cell receptor diversity [51]. Previous studies have shown significant oligoclonality of T cells in multiple primary immunodeficiencies, autoimmune diseases, and certain malignancies.

### *Severe Combined Immunodeficiency (SCID)*

SCID, the most severe combined immune defect, exhibits a lack of T cells as well as a primary defect in B-cell numbers or secondary defect in B-cell function [52]. This defective immune system leaves the body prone to recurrent infections, chronic diarrhea, failure to thrive, and ultimately death if untreated. SCID affects all ethnic groups and occurs in one in 50,000 to 100,000 live births [53]. Confirmation of SCID requires sequence analysis of the suspected genes, but flow cytometry has aided in the diagnosis of SCID as the various genetic abnormalities associated with SCID have a phenotypic impact on B-, T-, and NK-cell numbers (Table 3).

More than 10 different phenotypes of SCID have been defined by their causative genetic defects. X-linked SCID due to deficiency of the common gamma chain of the interleukin-2, -4, -7, -9, -15, and -21 receptors is the most frequent type [54]. Deficiency of this receptor leads to $T^-/B^+/NK^-$ SCID, since these receptors are essential for NK- and T-cell development. A similar phenotype, although inherited in an autosomal recessive manner, is seen in Jak3 deficiency since this signaling molecule binds to the common gamma chain. The majority of the B cells is naïve, express surface IgM, and are non-functional because of the lack of T cells, but also since IL-21 is required for proper B-cell function [55]. Flow cytometry can detect the expression of the common gamma chain (CD132) or Janus Kinase 3 (Jak3), and sequence analysis can confirm a defect [56]. Deficiency of the interleukin-7 receptor or CD3 subunits results in $T^-/B^+/NK^+$ SCID. $T^-/B^-/NK^+$ SCID is caused by mutations in genes affecting DNA rearrangement or repair and include the following: recombination activating genes (*RAG*) 1 and 2; DNA cross-link repair 1C (*DCLRE1C*), which codes for the Artemis protein; DNA ligase-4 (*LIG4*); protein kinase, DNA-activated, catalytic polypeptide (*PRKDC*), which codes for DNA-dependent protein kinase (DNA-PK); and non-homologous end-joining factor 1 (*NHEJ1*), which codes for protein cernunnos. The lack of the enzymes adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP) is another frequent cause of SCID due to the buildup of metabolites which are toxic to lymphocytes. Patients with ADA/PNP deficiency typically present with $T^-/B^-/NK^-$ SCID, although some may have normal levels at birth if there is residual enzyme activity, but these numbers decrease over time [57]. Other than the traditional analysis of dATP concentration and ADA activity in washed red cells, others have shown the ability to detect intracellular ADA using flow cytometry [58].

**Table 3** Various clinical phenotypes seen in SCID-related[a]

| Phenotype | Disease Name |
|---|---|
| $T^-/B^+/NK^-$ | • X-linked SCID (IL-2 receptor gamma deficiency) |
| | • JAK3 deficiency |
| $T^-/B^-/NK^+$ | • RAG-1 or RAG-2 deficiency |
| | • Artemis (*DCLRE1C*) deficiency |
| | • Ligase 4 (*LIG4*) deficiency |
| | • DNA-PK (*PRKDC*) deficiency |
| | • Cernunnos (*NHEJ1*) deficiency |
| $T^-/B^+/NK^+$ | • IL-7 receptor alpha deficiency |
| | • CD3 (gamma, delta, epsilon) deficiency |
| | • CD45 deficiency |
| | • ZAP70 deficiency |
| | • Coronin-1A deficiency |
| $T^-/B^-/NK^-$ | • ADA deficiency |
| | • PNP deficiency |
| | • Reticular dysgenesis (AK2 deficiency) |

[a] Adapted from 2012 IUIS Classification[1]

*SCID* severe combined immunodeficiency; *JAK3* Janus kinase 3; *RAG* recombination activating genes; *DCLRE1C* DNA cross-link repair 1C; *LIG4* ligase 4, DNA, ATP-dependent; *DNA-PK* DNA-dependent protein kinase; *PRKDC* protein kinase, DNA-activated, catalytic polypeptide; *NHEJ1* non-homologous end-joining 1; *ADA* adenosine deaminase; *PNP* purine nucleoside phosphorylase; *AK2* adenylate kinase 2

### *Omenn Syndrome*

Omenn syndrome is a variant of SCID with a characteristic erythroderma, eosinophilia, elevated IgE levels, lymphadenopathy, and hepatosplenomegaly. Omenn is classically caused by hypomorphic mutations in the *RAG1* or *RAG2* genes, but can be observed with other gene defects. Immunophenotyping reveals a low/absent B-cell count and T cells expressing a predominant memory phenotype (i.e., $CD45RO^+$) due to expansion of cells that escape the defect [59] (Fig. 3). Also, an increased percentage of γδ T cells have also been reported in these patients, and T-cell repertoire analysis will show a restricted pattern of T-cell receptors [60].

### *Major Histocompatibility Complex Class II Deficiency*

This rare autosomal recessive disease results in the loss of expression of the major histocompatibility complex (MHC) class II proteins. These proteins are normally found on antigen-presenting cells and thymic epithelium and are required for the development of $CD4^+$ T cells. The MHC class I protein expression and T-cell receptor expression is typically preserved [61]. Interestingly, MHC class I expression can be

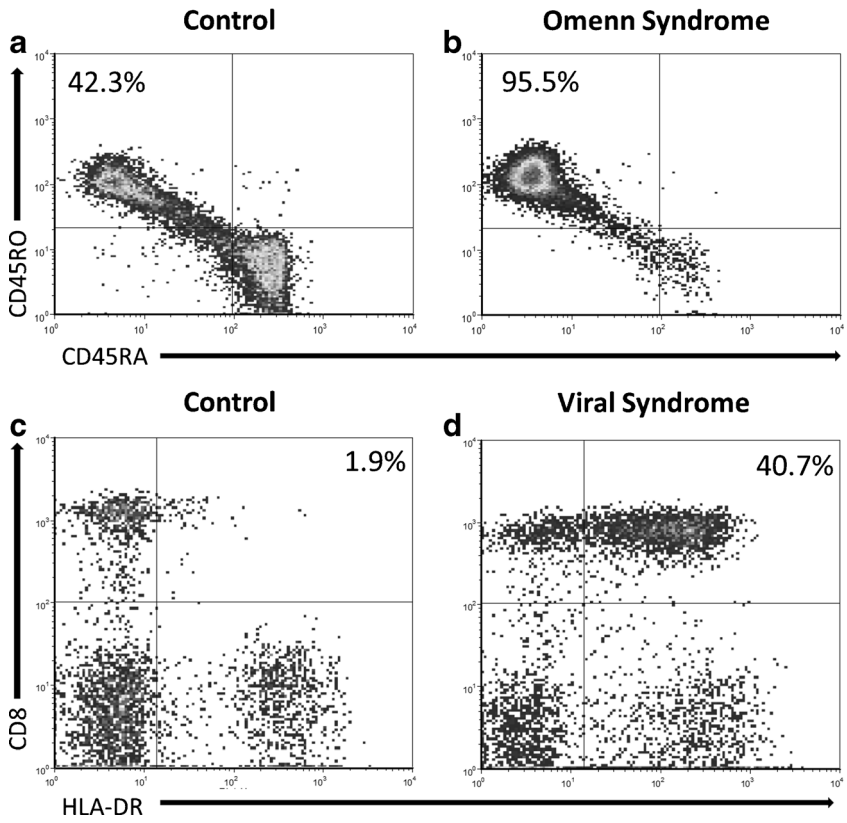


**Fig. 3** Flow cytometric analysis of T-cell activation and memory. Whole blood was analyzed with antibodies to CD4, CD45RA (naïve), CD45RO (memory), and HLA-DR. **a** Normal $CD4^+$ $CD45RA/RO^+$ profile. **b** Skewed $CD4^+$ $CD45RA/RO^+$ profile in Omenn syndrome. **c** Normal pattern of HLA-$DR^+$ expression in $CD8^+$ T cells in a healthy control. **d** Evidence of $CD8^+$ T cell activation based on HLA-$DR^+$ expression in patient with underlying immune defect and/or viral infection

affected, but this protein can be induced upon activation unlike the class II genes [62]. Immunophenotyping of these patients demonstrates normal numbers of both $CD8^+$ T cells and B cells, with reduced or absent $CD4^+$ numbers [63]. The B cells express high levels of IgM and IgD, with no detectable MHC class II proteins (HLA-DR, HLA-DP, HLA-DQ, or HLA-DM) which can be detected by flow cytometry [57].

*Wiskott-Aldrich Syndrome*

The triad of recurrent infections, thrombocytopenia, and eczema is synonymous with Wiskott-Aldrich syndrome (WAS). With increasing age, these patients demonstrate dwindling numbers of both T and B cells with varying $CD4^+/CD8^+$ ratios [33]. Platelet size in these patients is smaller than normal. This X-linked recessive disease is caused by a mutation in the WAS protein (WASp). Flow cytometric detection of WASp can rapidly screen for this disease. Presence of WASp, however, does not exclude the diagnosis, and sequencing analysis should be sent if WAS is suspected despite normal protein expression. Interestingly, there has been reported WASp mutation reversions in 11 % of WAS patients whose cells were previously WASp-negative [64]. It is believed that this reversion phenomenon may allow for the possibility of gene therapy as a potential treatment option in the future.

*DiGeorge Syndrome*

DiGeorge syndrome, otherwise known as 22q11 deletion syndrome, is characterized by thymic aplasia/hypoplasia which results in decreased T cells. Flow cytometry reveals decreased $CD3^+$, $CD4^+$, and $CD8^+$ T cells, with decreased $\alpha\beta$ T cells and normal numbers of $\gamma\delta$ T cells [28]. One may also see an increased $CD4^+/CD8^+$ ratio, but this will typically trend toward normal with age [65]. Deletions at 22q11 can be detected by fluorescent in situ hybridization (FISH) utilizing a *HIRA* (*TUPLE1*) probe which has a false-negative rate of approximately 5 % [66]. A newer method of mutation detect utilizes quantitative real-time PCR which has shown as sensitivity and specificity of 100 % detection, as well as decreased turnaround time [67].

*Hyper IgM Syndrome*

Hyper IgM syndrome (HIGM) is associated with defects in B-cell class switching and somatic hypermutation, which leads to normal/high IgM levels, but diminished levels of IgA and IgG. The impaired specific antibody function leads to rising IgM levels with infection or immunization and little IgG formation. Aside from the presentation of recurrent infections, often these patients can display manifestations of autoimmunity, malignancy, neutropenia, and combined cell-mediated

and humoral defects [68]. The most prevalent cause of hyper IGM syndrome the X-linked form due to a deficiency in CD40 ligand (CD40L, CD154). CD40L on activated T cells binds to CD40 on B cells and initiates B-cell isotype switching [69]. CD40 deficiency presents similarly, but is autosomal recessive. In addition to defects in class switching, CD40 is also important for activation of phagocytes (e.g., monocytes, dendritic cells), and these patients also suffer from opportunistic infections (e.g., *pneumocystis*, *cryptosporidium*, etc.). Flow cytometry has the capability to evaluate for the expression of CD40 ligand in activated CD4 T cells in cases of suspected X-linked HIGM [70] (Fig. 4). Monoclonal antibodies to CD40L have a detection sensitivity of 68 % in confirmed X-linked HIGM patients [71]. This sensitivity can be enhanced to 90 % by using a biotinylated CD40-Ig fusion protein which binds to a functional CD40L receptor complex [72]. Flow cytometry can be used as a screening test for the AR form of HIGM by evaluating for the absence of CD40, which is constitutively expressed on B cells, monocytes, and dendritic cells [73]. Two other forms of autosomal recessive HIGM need to be evaluated by genetic analysis, namely uracil nucleoside glycosylase (*UNG*) and activation-induced cytidine deaminase (*AID*) deficiency [74]. These result in hyper IgM but do not have associated cell-mediated defects.

## Disorders of Neutrophils

Neutrophil-related disorders typically present as recurrent skin and respiratory tract infections due to either bacteria or fungi (especially *Candida* and *Aspergillus*). Patient can also experience delayed umbilical cord separation, omphalitis, deep-seeded abscess formation, poor wound healing, and recurrent oral stomatitis.

Initial screening for these disorders should begin with CBC and differential to evaluate the absolute neutrophil count (ANC) and morphological analysis of neutrophils. A high ANC can be seen in response to infections, as well as in certain disorders of leukocyte adhesion. A low or absent ANC is seen in defects involving neutrophil development or maturation.

### *Leukocyte Adhesion Deficiency*

Leukocyte adhesion deficiency (LAD) typically presents with recurrent bacterial infections with no pus formation. One may also denote a history of delayed umbilical cord separation or omphalitis. There are three different groups of LAD diseases which are based upon their genetic abnormality and clinical characteristics. LAD type 1 is the result of a mutation in the β2-integrin protein CD18, which is shared by a number of integrin molecules, including leukocyte function-associated protein (LFA-1 or CD11a/CD18), Mac-1 (CD11b/CD18), and p150/95 (CD11c/CD18). The diminished levels of the CD11 or CD18 expression by flow cytometry is a diagnostic screen for LAD type 1 [73]. LAD type 2 is due to a defect in fucose metabolism, which results in the absence of sialyl lewis X (CD15s) which is a carbohydrate ligand on the cell surface of neutrophils that binds to the E- and P-selectins on activated endothelial cells. Abnormal CD15s expression on neutrophils by flow cytometry is indicative of LAD type 2. LAD type 3 is characterized by increased bleeding risk, recurrent infections, and leukocytosis. Unlike in LAD type I, the β2-leukocyte integrin are expressed; however, in LAD type 3, the integrins fail to function properly. LAD3 diagnosis requires specialized testing of integrin function in platelets or leukocytes or by molecular methods [35].

### *Chronic Granulomatous Disease*

Patients with chronic granulomatous disease (CGD) typically present with recurrent bacterial and fungal infections. Granulomatous inflammation occurs due to failure to clear the infections, and also due to an inherent propensity for increased inflammation in these patients. This clinical picture is due to a lack of neutrophilic nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which is made up of one X-linked gene and three autosomal genes. The X-linked gene is cytochrome b-245, beta polypeptide (*CYBB*, which codes for $gp91^{phox}$). The three autosomal genes include the following: cytochrome c-245, alpha polypeptide (*CYBA*, which codes for $p22^{phox}$), neutrophil cytosolic factor 1 (*NCF1*, which

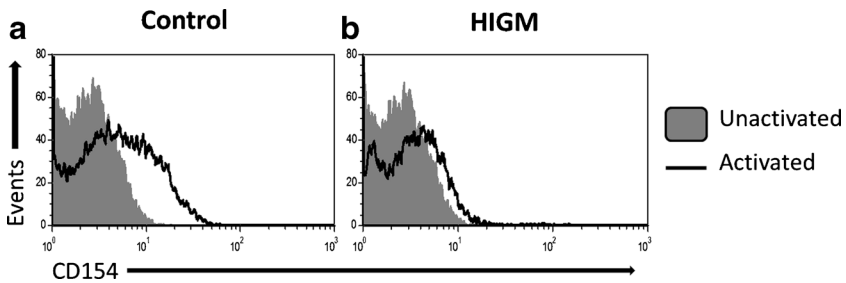


**Fig. 4** CD40L expression in Hyper IgM syndrome. Peripheral blood cells are activated pharmacologically and CD40 ligand (CD154) expression measured. **a** Comparison of CD40L expression in a control sample, unactivated (filled histogram), and activated peripheral blood (open histogram). **b** Lack of CD40L expression after activation, as seen in cases of HIGM. *HIGM* hyper IgM syndrome, *CD40L* CD40 ligand

codes for $p47^{phox}$), and neutrophil cytosolic factor 2 (*NCF2*, which codes for $p67^{phox}$). The absence of this enzyme complex results in a decreased/absent oxidative burst and production of reactive oxygen intermediates. Rapid flow cytometric analysis of a neutrophil oxidative burst can be done using the dihydrorhodamine 123 (DHR) assay (Fig. 5). In this assay, neutrophils are incubated with DHR and then stimulated with phorbol myristate acetate (PMA) resulting in a neutrophilic oxidative burst which oxidizes the DHR dye resulting in fluorescence. This assay is much more sensitive than the previously utilized colorimetric assay using nitroblue tetrazolium (NBT) and is not as prone to operator error. The DHR is typically abnormal in cases of CGD. In addition, the DHR assay can be used to determine the carrier state of mothers (and maternal relatives) of boys affected with the X-linked form since random X-chromosome inactivation results in half of the neutrophils failing to produce fluorescence (Fig. 5).

### *Glucose-6-Phosphate Dehydrogenase Deficiency and Myeloperoxidase Deficiency*

There are also two well-described genetic defects which result in presentations similar to CGD, myeloperoxidase deficiency and glucose-6-phosphate dehydrogenase (G6PD). Myeloperoxidase (MPO) plays a role in the bactericidal formation of reactive oxidative intermediates. Interestingly, more than 95 % of MPO-deficient patients are asymptomatic as in vitro testing has shown that MPO-deficient neutrophils retain killing potential, but at a slower rate [75]. The definitive diagnosis of this disease is by histochemical staining for MPO. Due to the functional nature of the enzyme, a DHR assay is also a reasonable testing option to consider; however, it can be falsely negative in cases of complete MPO deficiency [76]. For this reason, histochemical staining is preferred.

G6PD deficiency is an X-linked disease that rarely causes an impaired neutrophilic respiratory burst. This defect can occur when there is less than 5 % enzymatic activity in neutrophils and is overcome when there is greater than 20 % activity [77]. This disease can also be detected by DHR, but needs to be distinguished from CGD by evaluation of G6PD activity, especially in patients with chronic anemia and recurrent infections.

### *Hyper IgE Syndrome*

Hyper IgE syndrome (HIES) is characterized by recurrent *S. aureus* infections of the skin and pulmonary tract, high IgE, eosinophilia, eczema, and mucocutaneous candidiasis [78]. HIES occurs in an autosomal dominant nature due to STAT3 deficiency and an autosomal recessive nature due to DOCK8 deficiency. A majority of patients with HIES have a heterozygous, dominant-negative mutation in *STAT3* which is critical for inducing RORγt, the Th17 determining transcription factor [79]. The mechanism of *DOCK8* mutations is not

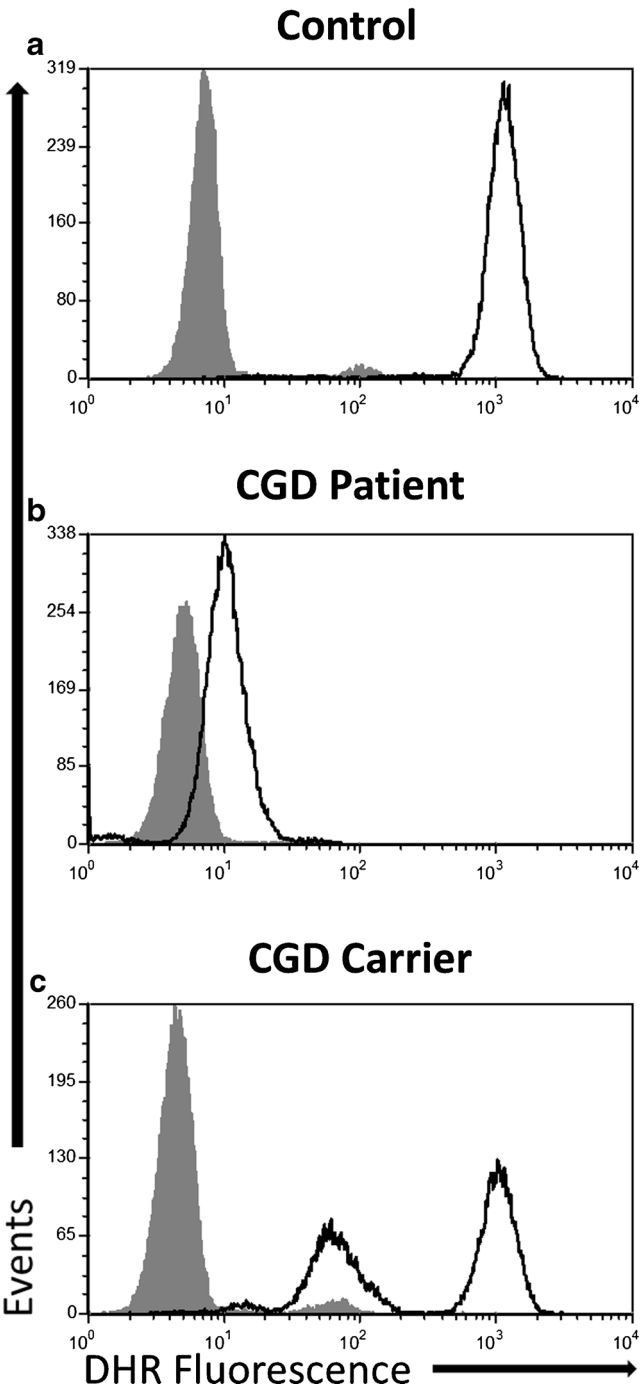


**Fig. 5** Evaluation of neutrophil oxidative burst in chronic granulomatous disease. Neutrophils are loaded with DHR then cultured in media alone (filled histogram) or activated with PMA (open histogram). **a** Normal activated neutrophils produce superoxides that oxidize DHR resulting in increased fluorescence as depicted by shift of histogram peak to the right. **b** CGD patients cannot generate oxidative burst and, therefore, do not oxidize DHR. **c** CGD carriers (usually mothers of affected male patients) demonstrate bimodal induction of neutrophil oxidative burst due to random X inactivation. *DHR* dihydrorhodamine-123, *PMA* phorbol 12-myristate 13-acetate, *CGD* chronic granulomatous disease

entirely understood, but patients do have defective $T_H17$ cell differentiation; however, it is different than STAT3 deficiency as RORγt expression is intact [79]. In particular, *STAT3* mutations also lead to connective tissue, skeletal, and vascular defects, whereas *DOCK8* mutations develop cutaneous viral infections (warts) and have a predisposition to malignancies at a young age [80]. In both conditions, a decrease in IL-17-producing T cells ($T_H17$) has been demonstrated. $T_H17$ cells play a role in autoimmunity and defense of extracellular pathogens (i.e., fungi, bacteria, and parasites) [81]. Although these diseases can be screened by evaluating the percentage of Th17 cells in the peripheral blood by flow cytometry, genetic mutational analysis is necessary for a definitive diagnosis.

## Natural Killer and Cytotoxic T-Cell Defects

Natural Killer (NK) cells play a key role in defending against viral infections. Defects in NK cell numbers typically present with recurrent, severe herpetic infections, while defects in NK and cytotoxic T lymphocyte (CTL) function result in the clinical phenotype of hemophagocytic lymphohistiocytosis (HLH) [82]. If there is concern for this type of immunodeficiency, screening should begin with immunophenotyping to confirm the presence or absence of NK cells.

Other than using immunophenotyping to evaluate for the presence of NK cells and cytotoxic T cells, it is also important to test their effector function. This may be done by performing a cytotoxicity assay which involves culturing labeled target cells with peripheral blood mononuclear cells (PBMCs), then measuring markers of cell death such as release of radiolabeled chromium or flow cytometric analysis of markers of apoptosis (i.e., annexin V, 7-ADD) [83]. Flow cytometric analysis of CD107a mobilization, a marker of degranulation, as well as evaluation of the presence of the intracellular cytotoxic proteins (i.e., perforin/granzyme) can be helpful in the evaluation of patients with these clinical characteristics [84-86]. Lysosome-associated membrane protein-1 (LAMP1 or CD107a) is normally expressed on the internal membrane of cytotoxic cell granules, which contain perforin and granzyme. These granules are transported to the cell surface and fuse with the target cell in order to release their contents. Perforin leads to pore formation and osmotic lysis, whereas granzymes induce apoptosis [87]. Defective CD107a expression has been used as a biomarker for disorders of degranulation, including familial HLH, Chediak-Higashi, and Griscelli syndrome [88, 89] (Fig. 6). Analysis of intracellular perforin is a rapid manner to detect for deficiency of this protein in cases of suspected HLH. It should be noted that infection, illness, or medication can affect NK-cell functional assays [90].

### *X-Linked Lymphoproliferative Syndrome*

The presentation of individuals with X-linked lymphoproliferative syndrome (XLP) includes fatal hemophagocytosis, hypogammaglobulinemia, or lymphoma [91]. Severe, even fatal infectious mononucleosis occurs in 2/3 of all XLP patients [92]. There are two different forms of XLP which are caused by two distinct genetic mutations. XLP-1 accounts for approximately 60 % of XLP cases due to mutation in SH2 domain containing 1A (*SH2D1A*), a signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) [91]. Immunophenotyping is able to demonstrate decreased/absent numbers of invariant natural killer T cells in XLP-1 [93]. The second form, XLP-2, is due to a mutation in the X-linked inhibitor of apoptosis gene (*XIAP*, also known as *BIRC4*) [92]. Flow cytometry can be used to detect intracellular SAP or XIAP expression [94].

## Adaptive-Innate Immunity Defects

### *Inherited Susceptibility to Mycobacterial Disease*

Patients with a Mendelian susceptibility to mycobacterial disease (MSMD) have an underlying problem involving the IL-12/INF-γ pathway and are vulnerable to mycobacterial infections and salmonellosis. Flow cytometry is able to evaluate for the presence of interleukin 12 receptor, beta 1 (IL12RB1), and/or interferon gamma receptor 1 (IFNGR1). The sensitivity of screening for the specific cell surface receptors by flow cytometry approaches 95 % in cases of IL12RB1 deficiency. Defects with *IFNGR1* occur in two varieties, autosomal dominant and autosomal recessive forms. Patients with the autosomal dominant form can present with osteomyelitis and have a noted over-expression of a non-functioning IFNGR1 protein when compared to controls [95]. Conversely, the autosomal recessive form of *IFNGR1*-related disease demonstrates an absence of IFNGR1 protein [96]. Another way one can evaluate these pathways is by measuring phosphorylated STAT1 after IFN-γ stimulation and phosphorylated STAT4 by IL-12 stimulation [97]. Decreased STAT1 phosphorylation would suggest that there is a possible defect in either *STAT1*, *IFNGR1*, or *IFNGR2*. Decreased STAT4 phosphorylation can occur in cases of IL-12 receptor deficiency [98] (Fig. 7).

## Disorders of the Complement System

The complement system is important in the control of bacterial infection and in the clearance of immune complexes generated during an immune response. Disorders of the complement system present with characteristic clinical pictures. Individuals who have a defect in the early complement system (C1, C2, C3, and C4) have an

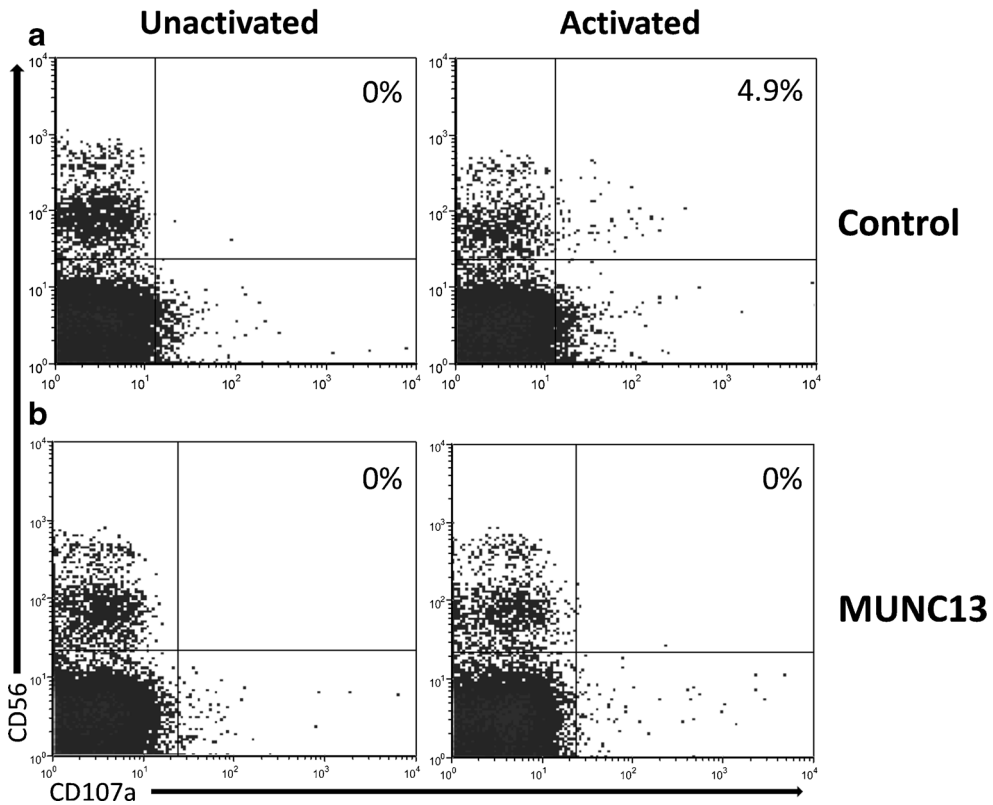


**Fig. 6** Evaluation of activated NK-cell lysosomal-associated membrane protein-1 (LAMP-1)/CD107a expression. NK cells are evaluated prior to (unactivated) and after co-culture with K562 erythroleukemic cell line (activated). Percentage shown is percent of NK cells expressing CD107a. **a** Inducible expression of CD107a on surface of $CD56^{+}$ NK cells in a healthy individual. **b** No upregulation of CD107a on NK cells of a patient with a MUNC13 mutation (FHLH3). *NK* natural killer, *MUNC13* mammalian uncoordinated-13, *FHLH3* familial hemophagocytic lymphohistiocytosis type 3

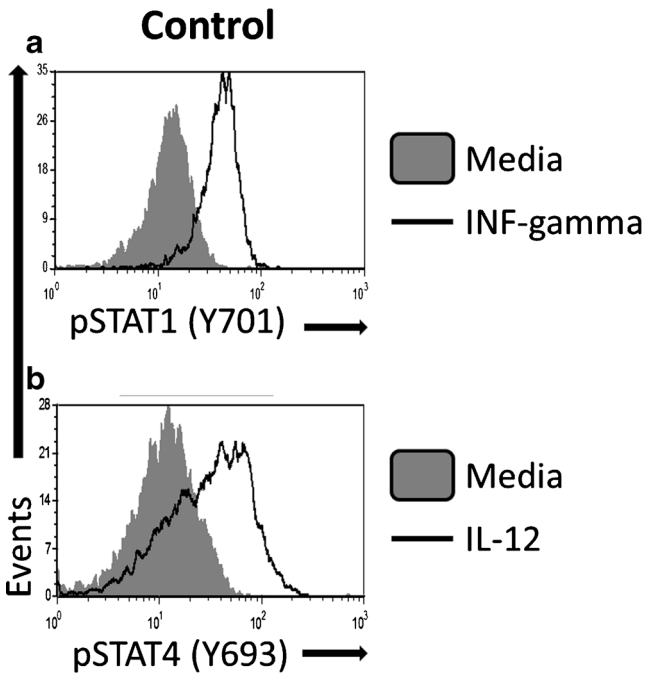


**Fig. 7** Evaluation of STAT1 and STAT4 defects in Mendelian susceptibility to mycobacterial disease. Peripheral blood mononuclear cells are stimulated in vitro with IFN-γ or IL-12 and phosphorylated STAT1 and STAT4, respectively, are measured. **a** Normal phosphorylation of STAT1 (Y701) in response to IFN-γ stimulation in monocytes. **b** Normal phosphorylation of STAT4 (Y693) in response to IL-12 in PHA-blasted lymphocytes. *STAT1* signal transducers and activators of transcription 1, *STAT4* signal transducers and activators of transcription 2, *IFN-γ* interferon gamma, *IL-12* interleukin-12, *PHA* phytohemagglutinin

increased risk of pyogenic infections and autoimmune diseases, in particular systemic lupus erythematosus. Those with defects in the late complement system (C5-9) result in susceptibility to neisserial infections, in particular *N. meningitidis*.

Screening for disorders of the complement system can be assessed by two laboratory tests, the CH50 and AH50. The total hemolytic complement (CH50) test measures the function of the classic complement cascade, whereas the alternative pathway (AH50) test measures the function of the alternative complement pathway. Patients with C1, C2, or C4 deficiency will have a low CH50, but normal AH50. Patients with a low AH50, but normal CH50, suggest a deficiency of factor B, factor D, or properdin [99]. A decrease in both CH50 and AH50 suggests a deficiency in a shared complement component, C3 or C5-C9 [96]. In order to confirm a suspected complement defect, one can assess individual complement levels or function.

## Immune Dysregulation Disorders

### *Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked Syndrome (IPEX)*

IPEX, an X-linked disease characterized by eczematous dermatitis, enteropathy, and endocrinopathies, is the prototypical presentation of an immune dysregulation disorder [100]. The

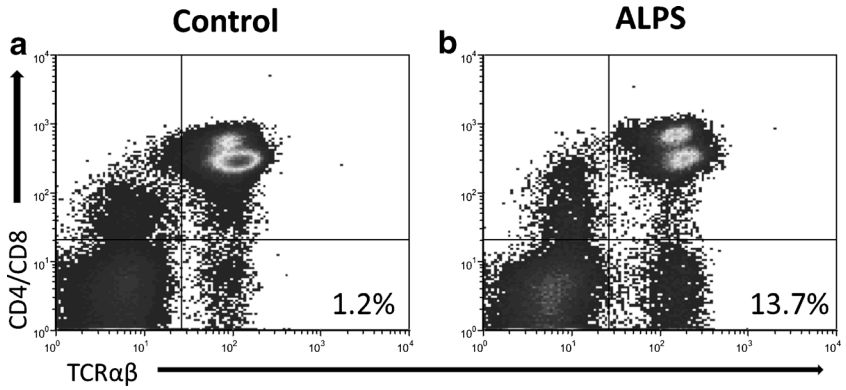


**Fig. 8** Evaluation of double-negative T cells in autoimmune lymphoproliferative syndrome. Normally, less than 2 % of TCR$\alpha\beta^+$ T cells do not express either the CD4 or CD8 co-receptors. These DNTs increase in number in ALPS. **a** Detection of DNT cells in a normal individual. **b** Increased DNT cells in a patient with ALPS. *TCR$\alpha\beta$* T cell receptor alpha/beta, *DNT* double-negative T cells, *ALPS* autoimmune lymphoproliferative syndrome

dermatitis is typically eczematous in nature, but exfoliative dermatitis, psoriasis-like lesions, and pemphigus nodularis have been seen [101]. The enteropathy typically manifests as profuse watery diarrhea within the first few months of life. Intestinal biopsies have noted villous atrophy [102]. The most common endocrinopathy is type 1 diabetes, but thyroid disease is also common [103]. The immune dysregulation in these patients can also lead to significant autoimmune phenomena, most frequently immune-mediated cytopenias. This disease is caused by defects which affect the forkhead box P3 (Foxp3) protein [104]. Interestingly, only about 50 % of patients with an IPEX phenotype have FOXP3 gene mutations [105]. Foxp3 is involved in the function of regulatory T cells which help control autoreactive T cells [106]. A CBC may show eosinophilia, anemia, neutropenia, thrombocytopenia, or subsequent immunophenotyping can be relatively normal. Quantitative immunoglobulins may be normal, elevated, or low due to severe protein-losing enteropathy. IgE levels are typically elevated, which may relate to severe food allergies noted in some of these patients [107]. Flow cytometry can be used to identify Foxp3-expressing $CD4^+$ T cells [108]. However, expression of Foxp3 is not sufficient to rule out IPEX, and sequence analysis of the FOXP3 gene should be evaluated if the clinical picture is consistent. Other proteins which affect regulatory T-cell development and function, such as CD25 or STAT5 deficiency, can also result in an IPEX-like phenotype [109, 110].

### *Autoimmune Lymphoproliferative Syndrome*

Autoimmune lymphoproliferative syndrome (ALPS) typically presents with lymphadenopathy, hepatomegaly, and autoimmune cytopenias [111]. Although the lymphoproliferation associated with this disease is non-malignant, patients do carry an increased risk of developing Hodgkin's or non-Hodgkin's lymphoma [112]. This syndrome is caused by mutations in genes which induce lymphocyte apoptosis. These mutations occur in the genes encoding for FAS (CD95), FAS ligand, and caspase 10 [113]. One diagnostic criteria for this disease includes an increased percentage of double-negative T cells (DNT, $CD3^+CD4^-CD8^-$ TCR$\alpha\beta^+$) [113] (Fig. 8). These cells also express B220 and CD27 [114]. Other significant findings include decreased $CD4^+CD25^+$ T cells, expanded $CD3^+$HLA-$DR^+$ T-cell population, decreased levels of $CD27^+$ B cells, increased $CD5^+$ B-cell count, increased $CD8^+CD57^+$ T-cell numbers, and hypergammaglobulinemia [115]. Serum levels of IL-10, IL-18, or vitamin B12 can be elevated in a peripheral blood sample [113].

## Conclusion

Laboratory evaluation of primary immunodeficiencies is complex but essential to the care of these patients. Stepwise, systematic testing is important to reach a correct diagnosis without unnecessary testing. Through this tiered testing approach, clinicians will be able to provide appropriate and directed decisions that will benefit their patients. Much like the testing decisions, the interpretation of these results should always be placed within the context of the patient and their current clinical scenario.

There have been many new innovations in immunodiagnostic studies over the last several years with new protocols on the horizon. Current diagnostic laboratories are no longer limited to evaluating cellular markers, but are now able to perform diverse functional assays. As the knowledge of the immune system expands, new testing will become available to aid in the diagnosis of primary immunodeficiencies.

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