Chapter

Flow Cytometry Applied to the Diagnosis of Primary Immunodeficiencies

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Abstract

Primary immunodeficiencies are the result of biological defects associated with functional immune abnormalities. It consists of a group of disorders showing a higher incidence and severity of infections, expression of immunological dysregulation such as inflammation and lymphoproliferation. The immunophenotyping and *in vitro* functional characterization of immunodeficient patients contribute, together with the clinical aspects, to define the underlying immune defect particularities. Flow cytometry applications in primary immunodeficiency assessment are multiple and include the study of a wide range of specific cell lymphocyte subpopulations. This chapter describes the main techniques used in the diagnosis of a wide variety of primary immunodeficiencies, in which intracellular proteins or activation markers involved in immunity are evaluated, as well as functional proliferation, cytokine production, phosphorylation of transcription factors, cytotoxic and degranulation capacity. Flow cytometry is a tool that allows rapid and accurate evaluation of multiple lymphocyte populations and immunological function, and this information is essential for the diagnosis and evaluation of patients with primary immunodeficiencies.

Keywords: primary immunodeficiencies, lymphocyte subsets, extended immunophenotyping, flow cytometry, functional assays, intracellular cytokine production, cytotoxicity, phospho-stat flow

1. Introduction

Primary immunodeficiencies (PID) are a heterogeneous group of genetic disorders which manifest clinically as recurrent infections, autoimmune and/or autoinflammatory diseases or malignancies. In PIDs, the immune system is affected quantitatively or qualitatively and includes more than 400 different entities, with an incidence of approximately 1:2000 live newborns. The Expert Committee of the International Union of Immunological Societies has classified PIDs into nine groups based on the clinical manifestations and laboratory immunological abnormalities [1]. Prompt identification of PID patients reduces complications and is associated with a more favorable prognosis [2]. In addition to a complete medical history, physical examination, and general laboratory tests, the initial evaluation protocol when PID is suspected includes analysis of serum immunoglobulin levels and extended immunophenotyping in peripheral blood. Immunophenotype abnormalities can range from a complete absence of a specific cell population to

more subtle variations in the differentiated states of specific subpopulations [3]. For basic and advanced lymphocyte phenotype studies, the technical option is classical flow cytometry, although new approaches mainly used in research like mass cytometry or CyTOF, have emerged [4]. The following step in the diagnosis of patients with PID is the biochemical and functional characterization of the altered molecules, for example, perforin, CD40 ligand, etc. The advantage of using flow cytometry over other techniques such as western-blot in the case of protein expression studies and functional evaluation of different subpopulations is the rapid and sensitive result.

2. Evaluation of immunophenotype to aid in the differential diagnosis of PIDs

Flow cytometry is a standard laboratory tool in the evaluation and identification of leukocyte populations and specific lymphocyte subpopulations. The analysis of the expression of cell surface markers [clusters of differentiation (CD)] allows to determine the cell lineage and to examine the stage of differentiation and cell activation. The clinical application is broad for routine use in diagnostic laboratories, which facilitates the assessment of patients with suspected primary immunodeficiencies. The circulating cellular compartment of the human immune system shows the most marked shifts during the first years of life, but is also dynamic during adult life, showing a tendency to a reduction in function and diversity with age [5]. When an immune system disorder is suspected, immunophenotyping of the patient's peripheral blood mononuclear cells (PBMCs) is one of the initial steps in the diagnostic work-up [6].

The basic immunophenotype analyzing T (CD3), B (CD19), and natural killer (NK) (CD56+CD16+) lymphocytes leads to a potential diagnosis of severe combined immunodeficiencies (SCID) resulting in four general categories of disease defined by the impact on lymphocyte populations: T−/B−/NK−, T−/B−/NK+, T−/B+/NK−, and T−/B+/NK+. Overall, patients with adenosine deaminase (ADA) deficiency and reticular dysgenesis demonstrate the T−/B−/NK− immunophenotype and typically have the most profound lymphocytopenia among SCID patients [7]. Recombination defects usually show the phenotype T−/B−/NK+, and defects in cytokine signaling and T-cell receptor (TCR)-derived signals result in T−/B+/NK− and T−/B+/NK+ phenotypes. These phenotypes often differ depending on the type of mutation and protein affected. The expression of isoforms CD45RA, CD45RO in T cells should also be included in the study of patients with suspected SCID. An increase in the percentage of CD45RO memory helper T cells indicates the presence of autoreactive lymphocytes and reinforces the diagnostic suspicion of SCID, leaky SCID or Omenn syndrome (OS) (**Figure 1**). OS is an inherited disorder characterized by an absence of circulating B lymphocytes and an infiltration of the skin by activated T lymphocytes. Inherited mutations in RAG1 or RAG2, resulting in partial V(D)J recombinase activity, were shown to be responsible for OS [8].

The use of larger cytometric panels can include a higher number of lymphocyte subpopulations of interest in the diagnosis of other groups of PID, such as predominantly antibody deficiencies and immune dysregulation diseases among others. Below we describe the gating strategies for the study of a broader spectrum of subpopulations: T cell differentiation stages, recent thymic emigrants (RET), regulatory T cells (Tregs), T helper 1/2/17 cells, B cell differentiation stages, NK cells, dendritic cells and monocytes, and their application in the study of patients with PID.

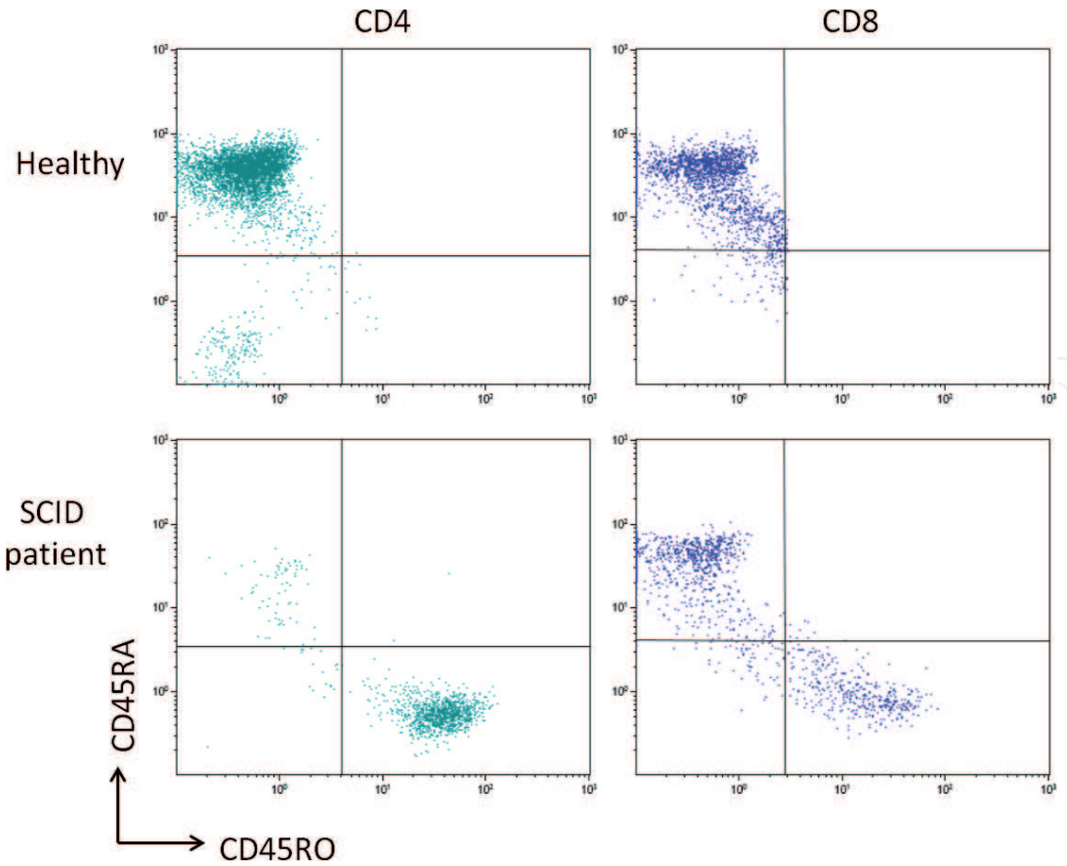

Figure 1.
Dotplot representing the expression of different isoforms CD45RA and CD45RO in subpopulations T CD4 and CD8. A healthy donor and a patient with severe combined immunodeficiency (SCID) of the same age range are compared.

General immunophenotyping protocol:

1. Stain 50 μL of EDTA whole blood with appropriate mix of antibodies of interest, depending on the panel.

2. Incubate for 20 min in the dark at room temperature (RT).

3. Lyse the red cells using a lysing solution.

4. Incubate for 10 min in the dark at RT.

5. Discard the lysing solution after 5 min of centrifugation at (324 g) or 1500 revolutions per minute (rpm).

6. Wash with 2 mL of phosphate buffered saline (PBS).

7. Discard the PBS after 5 min of centrifugation at 1500 rpm.

8. Add 200 μL of PBS, vortex and the sample is ready to be acquired by flow cytometry.

(This protocol is used in all the immunophenotyping techniques, using the appropriate mix of antibodies in each situation).

2.1 T lymphocyte immunophenotyping

T-lymphocytes mature in the thymus after going through positive and negative selection processes before migration into secondary lymphoid organs. The immune system at birth is largely dominated by truly naïve T cells, with very limited representation of memory cells and a relatively high proportion of recent thymic emigrants (RTEs). They are naïve peripheral T cells, which have only recently exited the thymus, have not undergone further peripheral proliferation and exhibit phenotypic and functional characteristics distinct from those of their more mature counterparts in the naïve peripheral T cell pool [9]. Thymic function can be determined by evaluation RTEs, which are studied using CD3, CD4, CD27, CD31, and CD45RA, expression markers according to the gating strategy in (**Figure 2**). The assessment of the thymic function is performed also in a suspicion of severe combined immunodeficiency, DiGeorge syndrome, and other T-cell immune deficiency disorders.

Subsequently, the mature "naïve" T-lymphocytes circulate between the secondary lymphoid organs and the blood. In humans, naïve CD4+ T cells typically express CCR7, CD62L, and CD45RA, while they lack CD45RO expression [10]. CCR7 and CD62L are involved in the localization of T cells in secondary lymphoid organs (SLO) and interact with ligands expressed in high endothelial venules (HEVs). CD45RA and CD45RO play a role in the transduction of TCR signals, and their expression characterizes the different subsets of T cells, being CD45RA positive the naïve and CD45RO positive the memory [11]. Upon recognition of specific antigen complexes, naive CD4+ T cells proliferate and differentiate toward effector T cells, which provide immediate protection.

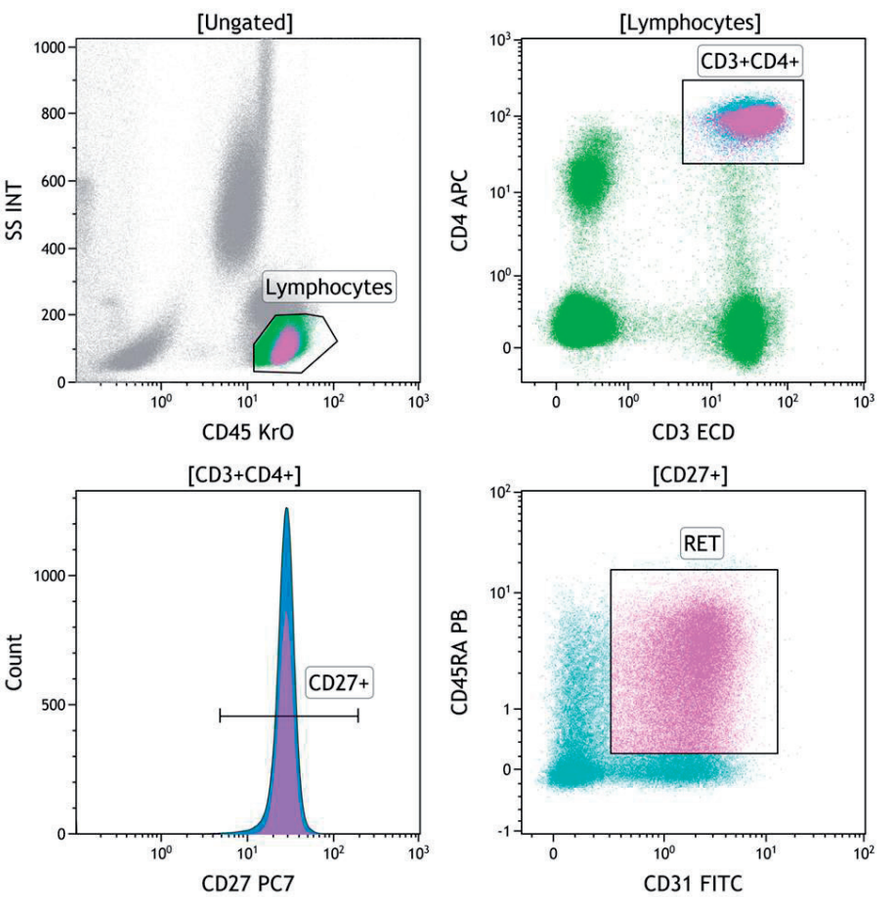

Figure 2.
Gating strategy of recent thymic emigrants (RET). RTEs were studied using CD3, CD4, CD27, CD31, and CD45RA expression.

Most of these effectors T cells subsequently die from apoptosis, but a subset of antigen-specific T cells will persist as memory T cells [12]. There are two types of memory T cells in circulation, central memory T cells (TCMs) and effectors memory T cells (TEM): the former shows self-renewal potential with less effector functions, while the latter possesses immediate effector functions and can migrate rapidly to peripheral tissues. In addition, a distinct lineage of tissue-resident memory cells (TEMRA cells) is limited to different tissues. By using the characteristic expression markers CD45RA and CCR7 and a gating strategy for gated CD4+ and CD8+ T-cell subsets, we can identify the following populations CD45RA+/CCR7+ (naïve), CD45RA−/CCR7+ [central memory (TCM)], CD45RA−/CCR7− [effector memory (TEM)], and CD45RA+/CCR7− [terminal effector memory (TEMRA)]. Furthermore, based on CCR6 and CXCR3 expression markers, we can distinguish CD4+ T-helper (Th) populations at phenotypic level (Th1, Th2, Th17, and Th1-17) by gating on CD4+CD45RA− TCM and TEM cells (**Figure 3**).

In several primary immunodeficiencies, there are alterations in different subpopulations. The distribution of naïve and memory T cells is particularly relevant for the diagnosis of severe combined immunodeficiency (SCID) and its variants in infants—Omenn syndrome and leaky SCID.

In patients with antibody defects such as common variable immunodeficiency (CVID) has been described an increase abnormally differentiated T CD4+ effector cells showing a Th1-skewed profile and activated effector T CD8+ lymphocytes [13, 14].

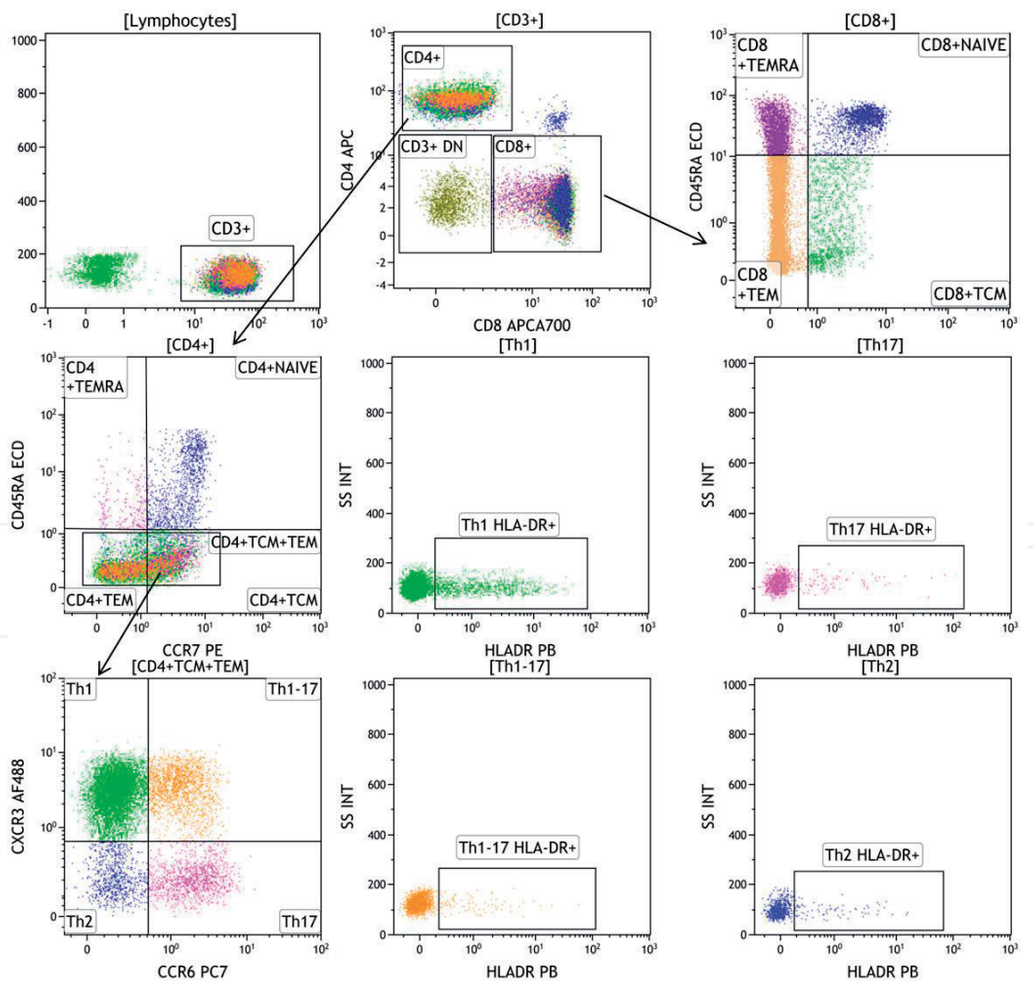

Figure 3.
Gating strategy for differentiated CD4+ and CD8+ T cell subsets, based on CD45RA and CCR7 expression defining: CD45RA+/CCR7+ (naïve), CD45RA−/CCR7+ (central memory [TCM]), CD45RA−/CCR7− (effector memory [TEM]), and CD45RA+/CCR7− (terminal effector memory [TEMRA]). CD4+ T-helper (Th) populations (Th1, Th2, Th17, and Th1-17), based on CCR6 and CXCR3 expression, were analyzed by gating on CD45RA− TCM and TEM cells.

Also with the suspected diagnosis of hyper-IgE syndrome (atopic dermatitis, recurrent otitis, pneumonias, and abscess caused by *Staphylococcus aureus*), the use of this gating strategy is very useful. The immunological phenotypes include high IgE levels, eosinophilia, low levels of Th17 cells, and memory B cell lymphopenia. T-cell differentiation, particularly Th17 cells is disrupted, for that reason, we use a flow cytometry panel to analyze the CD4+ T-helper (Th) populations (Th1, Th2, Th17, and Th1-17), based on CCR6 and CXCR3 expression, previously gated on CD45RA− TCM and TEM CD4+ T cells. It is important to compare the values of the patient with an age-matched healthy control [6].

Regulatory T-cells (Tregs) play a key role in maintaining peripheral tolerance under physiological and pathological conditions. Tregs are characterized by the constitutive expression of the critical transcription factor forkhead box P3 (Foxp3). Impairment of Treg function is the key pathogenic event leading to altered self-tolerance in patients with immune deregulation, poly-endocrinopathy, enteropathy, and X-linked syndrome (IPEX) [15]. The most common markers utilized for Treg typing by flow cytometry are CD25, forkhead box protein 3 (FoxP3), and CD127. Also for more accurate characterization, CCR4 is useful; a potential marker for effector-type regulatory T cells T-regulatory (Treg) cell populations: CD3+CD4+CD25+, CD127−, CCR4+, and CD45RO+.

See the immunophenotype protocol in Section 2.

2.2 B lymphocyte immunophenotyping

Early development of human B cells from multipotent stem cells occurs in the bone marrow by antigen independent manner. Once the central development of B cells has been accomplished and the transitional B cells leave the bone marrow, maturation occurs in the secondary lymphoid organs, especially in the spleen.

B-cell subset phenotyping in peripheral blood has been used to classify and assess the prognosis of patients with humoral deficiencies such as common variable immunodeficiency (CVID). CVID is a heterogeneous disease, and most patients show defective differentiation of B cells into memory B cells or antibody-secreting cells. B cells can be identified in the lymphocyte gate by CD19 expression, a marker that is present in all circulating B cell populations. B-cell populations can be studied based on expression of IgD and CD27 [naïve (IgD+CD27−), pre-switched (IgD+CD27+), switched memory (IgD−CD27+), and exhausted (IgD−CD27−)]. In patients with CVID, the study of these subpopulations allows to identify severe defects of germinal center dependent B cell memory formation and early defects of peripheral B cell differentiation. The absence of switched memory B cells is very suggestive of an alteration of the germinal center reaction [16].

The combined staining for CD19, IgD, CD38, and CD24 allows the identification of transitional B cells ($CD19+IgD+CD38^{high}CD24^{high}$) and plasmablasts ($CD19+IgD+CD38^{high}CD24^{neg}$). CD27 and CD21 enabled study of the $CD21^{low}$ population. The expansion of circulating $CD21^{low}$B cells is associated with manifestations of a chronic immune activation and autoimmunity (**Figure 4**) [17].

Recently, germline heterozygous gain-of-function mutations in PIK3R1 and PIK3CD genes have been identified to cause a primary immunodeficiency in patients with node lymphoproliferation, splenomegaly, diarrhea, and dysgammaglobulinemia, initially diagnosed with CVID. In these patients, the extended immunophenotyping reveals high percentage of transitional B cells, low pre-switched memory, switched memory B cells, and skewing of CD4+ with CD8+ T cells toward terminally differentiated effector cells. This phenotype is suggestive of activated PI3K delta syndrome type 1 and 2 (APDS) [18].

See the immunophenotype protocol in Section 2.

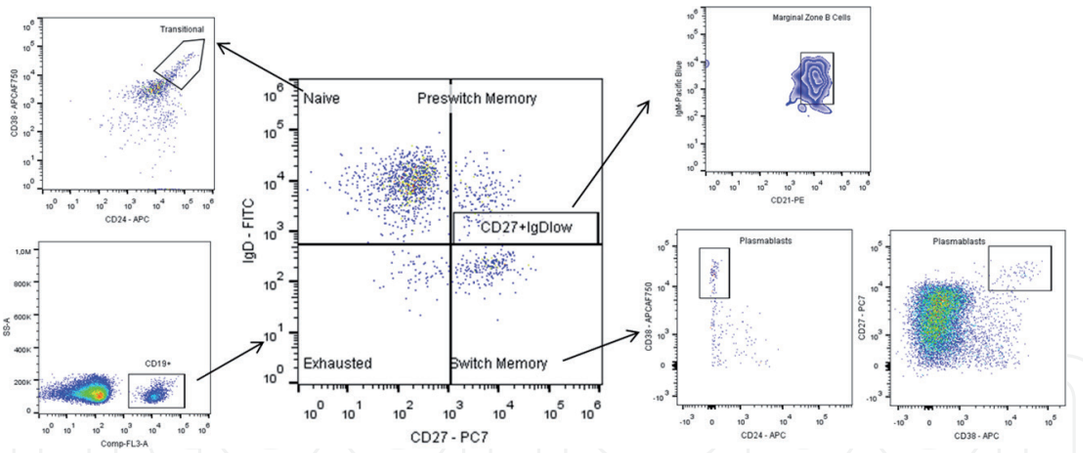

Figure 4.
Gating strategy used for the study of subpopulations of B lymphocytes. B-cell populations (naïve, pre-switched, switched memory, and exhausted) are distinguished depending on IgD and CD27 expression. The differing pattern of CD24+ and CD38+ expression identified transitional cells and plasmablasts. CD27 and CD21 markers enabled study of the CD21 low population.

2.3 Innate cells immunophenotyping

2.3.1 Natural killer (NK) cells

Natural killer (NK) cells are one of the first lines of innate immune defense against pathogens and tumors, among other cells. NK cells are lymphocytes with cytotoxic capacity that provide rapid response to virus-infected cells and to tumor cells through the ability to recognize the absence of HLA class I and other signals on these cells. Innate cells can be analyzed by flow cytometry gating in the $CD3^{-}CD19^{-}$ cells. Natural killer (NK) cells subpopulations can be divided into two subpopulations depending on CD56 and CD16 expression; NK^{dim} ($CD16^{+}CD56^{low}$) and NK^{bright} ($CD16^{-}CD56^{high}$). Characterizing the different subsets of NK is crucial in the diagnosis of NK cell deficiencies [19]. An example is the deficiency in MCM4, with autosomal recessive inheritance (AR), which may be suspected in the context of a higher percentage of CD56bright NK cells (of total NK cells) in individuals with endocrine and/or growth abnormalities [20].

See the immunophenotype protocol in Section 2.

2.3.2 Monocytes and dendritic cells (DC)

Monocytes are leukocytes that can differentiate into macrophages and myeloid lineage dendritic cells. As a part of the vertebrate innate immune system, monocytes also influence the process of adaptive immunity [21]. There are at least two subpopulations of peripheral monocytes in human blood based on their phenotypic receptors CD16 and CD14; classical monocytes ($CD14^{high}CD16^{-}$) and non-classical monocytes ($CD14^{low}CD16^{+}$). These cells have been shown to exhibit distinct phenotype and function [22].

Dendritic cells in periphery blood are responsible for the capture and processing antigens, express lymphocyte co-stimulatory molecules, migrate to lymphoid organs, and secrete cytokines to initiate immune responses [23]. Dendritic cells were studied into the population negative for the following markers: CD3, CD14, CD16, CD19, CD20, and CD56. High expression of HLA-DR, CD11c, and CD123 are markers commonly used to identify plasmacytoid DCs ($HLA\text{-}DR^{+}CD123^{+}$) and myeloid DCs ($HLA\text{-}DR^{+}CD11c^{+}$) (**Figure 5**). GATA binding protein 2 (GATA-2) deficiency is an autosomal dominant (AD) immunodeficiency classified in the

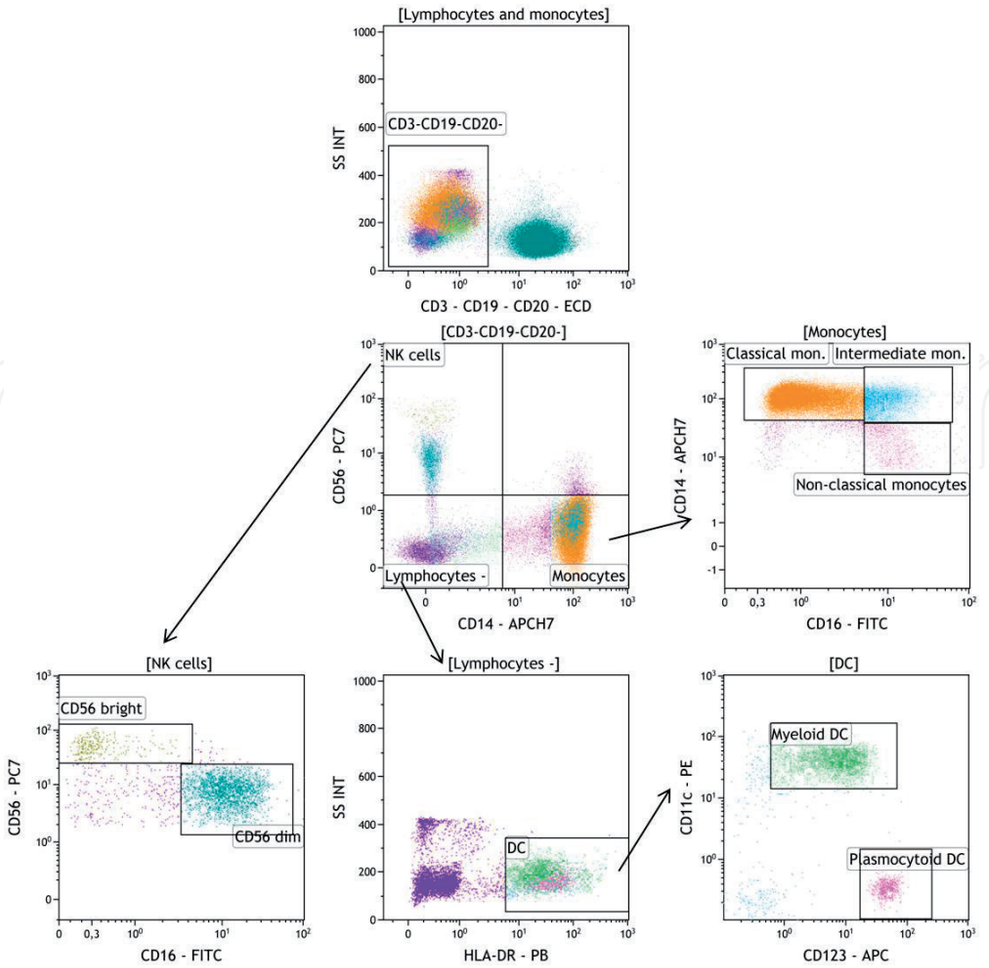

Figure 5.
NK/monocytes/DC dendritic cells (DC) gating strategy. NK cells and monocyte populations were analyzed in the CD3−CD19− gate. NK subpopulations (NK^{dim} and NK^{bright}) were studied using CD56 and CD16 expression markers. The markers CD16 and CD14 were used to identify classical monocytes (CD14+CD16−) and non-classical monocytes (CD16+CD14−). DCs were studied selecting the population negative for the following markers: CD3, CD14, CD16, CD19, CD20, and CD56. High expression of HLA-DR and CD11c and CD123 was used to identify plasmacytoid DCs (HLA-DR+CD123+) and myeloid DCs (HLA-DR+CD11c+).

group of congenital defects of the number or function of phagocytes in which the following leukocyte populations are affected: $CD56^{bright}$ NK cell, B lymphocytes, monocytes and peripheral dendritic cells populations are affected. Patients with this PID have susceptibility to infections by mycobacteria and human papillomavirus (HPV), histoplasmosis, alveolar proteinosis, myelodysplastic syndromes such acute myelogenous leukemia and lymphedema [24].

See the immunophenotype protocol in Section 2.

3. Evaluation of intracellular proteins and activation markers involved in immunity

3.1 Evaluation of intracellular proteins

Intracellular protein and specific cell surface antigen detection is a screening test for PIDs. Depending on the specific clinical presentation of the patient, the diagnostic suspicion can be assessed by evaluating the expression of the affected protein by flow cytometry. The most widely used procedure for staining intracellular molecules requires the fixation of cells in suspension and subsequent permeabilization

before adding the detection antibody. This fixation/permeabilization treatment allows the antibody to pass through the plasma membrane into the cell while maintaining morphological characteristics. While this technique is informative and very useful to identify total defects of a particular protein, detecting expression does not rule out the defect, as there may be mutations that do not alter the expression but the function. Some examples of this type of application to PID diagnosis are the evaluation of the following proteins:

For intracellular staining, there are several commercial options, including laboratory permeability and fixation reagents. It is important to take into account that not all commercial kits work the same way for different cell types or for different techniques. It is therefore necessary to adjust the conditions in each particular case.

Intracellular staining protocol:

1. Fix 50 μL of EDTA whole blood with the appropriate volume of fixative reagent.

2. Incubate for 15 min in the dark at RT.

3. Add the appropriate volume of permeabilizing reagent.

4. Add the extracellular antibodies mix.

5. Add the appropriate intracellular antibody.

6. Incubate for 20 min at RT and in the dark.

7. Add the appropriate volume of wash buffer.

8. Discard the wash buffer after 5 min of centrifugation at 1500 rpm.

9. Add 100 μL of PBS and the sample is ready to be acquired by flow cytometry.

(This protocol may change slightly depending on the commercial kit used).

3.1.1 BTK expression in X-linked agammaglobulinemia

X-linked agammaglobulinemia or Bruton tyrosine kinase (BTK) deficiency is a disorder caused for mutations in the gene encoding BTK. It is a tyrosine-protein kinase localized intracellularly next to the B-cell receptor (BCR), and it is crucial for the B cell development. The expression of BTK protein is evaluated in monocytes or platelets as patients with suspected X-linked agammaglobulinemia have an absent or significantly reduced number of circulating B lymphocytes. This leads to the fact that intracellular BTK protein has to be studied by gating monocytes [25].

See the intracellular staining protocol in Section 3.1.

3.1.2 SAP, XIAP, and perforin expression in Hemophagocytic lymphohistiocytosis (HLH)

Measurement of intracellular signaling lymphocyte activation molecule-associated protein and X-linked inhibitor of apoptosis are used as screening tools to evaluate X-linked lymphoproliferative syndrome disorder type 1 and 2, respectively. This approach has been shown to provide good sensitivity and specificity compared

with genetic mutation evaluation, and it is characterized by high negative predictive value. The lack of intracellular perforin expression in NK cells is characteristic of familial hemophagocytic lymphohistiocytosis type 2 [26, 27].

See the intracellular staining protocol in Section 3.1.

3.1.3 FOXP3 expression in IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked) syndrome

A further example of intracellular protein testing by flow cytometry in patients with PIDs involves screening for patients with possible immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (the gene encoding FOXP3 protein is located on the X chromosome, therefore only males are affected by the disease while females are carriers). This diagnosis is confirmed in male patients whose CD4+CD25+ regulatory T cells demonstrate absence or decreased expression of FOXP3 [28].

See the intracellular staining protocol in Section 3.1.

3.2 Immediate early activation markers

The activation of T lymphocytes is a complex and finely regulated process of events resulting in the expression of cytokine receptors, the production and secretion of cytokines and increased cell surface molecules that facilitate the immune response. The most important activation molecules expressed on T lymphocytes can be classified as early activation markers, such as CD69 and CD25, and late activation markers, such as CD62L and HLA-DR. Additionally, very late activation markers, such as VLA-1 have been described, playing a role in lymphocyte adhesion and extravasation [29].

CD69 is a signaling membrane glycoprotein involved in the induction of T cell proliferation. CD69 is expressed at very low levels T cells at rest in PBMCs (<5–10%) and is one of the first evaluable activation markers, being rapidly increased in T cells within the first hour of TCR. Expression of CD69 peaks at 16–24 h and then decreases. The inability to upregulate CD69 following TCR activation may be associated with T cell dysfunction [30].

CD40 ligand (CD40L), also called CD154, is a member of the TNF-receptor superfamily, primarily expressed on activated T cells, and functions as a co-stimulatory molecule by binding CD40 which is constitutively expressed on antigen presenting cells (APCs). The CD40L-CD40 ligation is a fundamental interaction for B lymphocytes proliferation, formation of germinal centers, Ig class switch, antibodies maturation, and B lymphocyte differentiation to plasmatic cell and to memory B lymphocyte. Deficiency of CD154 is an X-linked disorder that only males are affected and females are the carriers, also known as Hyper IgM syndrome [31]. Expression of CD40L on resting T cells from healthy donors is very low (<1%) and is quickly upregulated within 1–2 h after TCR stimulation via the transcription factors NFAT and AP-1. CD40L expression peaks near 6 h after stimulation and declines by 16–24 h (**Figure 6**) [32].

In summary, CD69 and CD40L are both rapidly induced following T cell activation and both exert important functions in T cell biology. Expressions of these markers have both been shown to be altered in patients with different PIDs.

Immediate early activation markers staining protocol:

1. Stimulate heparin whole blood or peripheral blood mononuclear cells (PBMCs) with the appropriate stimuli or mitogen.

2. Incubate the appropriate time depending on the activation marker of your interest.

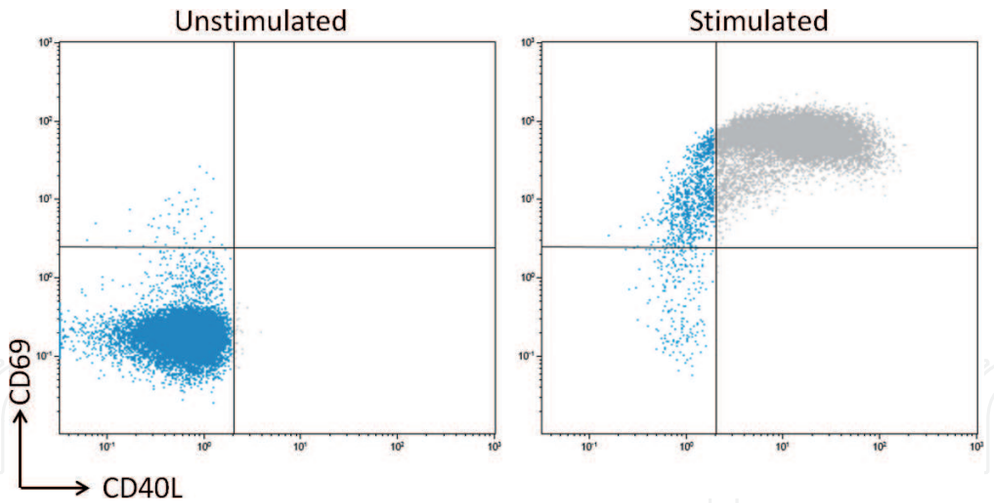

Figure 6.
The expression of CD40 ligand is studied by stimulating PBMCs with PMA plus ionomycin for 4 h, and then the expression of a cell activation marker such as CD69 is analyzed. Dotplots of PMBCs without stimulation (left) and stimulated (right) where a normal expression of CD40L and CD69 is observed.

3. Stain with appropriate mix of antibodies of interest.

4. Incubate for 20 min at RT and in the dark.

5. Lyse the red cells using 2 mL of lysing solution.

6. Incubate for 10 min at RT and in the dark.

7. Discard the lysing solution after 5 min of centrifugation at 1500 rpm.

8. Wash with 2 mL of PBS.

9. Discard the PBS after 5 min of centrifugation at 1500 rpm.

10. Add 100 μL of PBS and the sample is ready to be acquired by flow cytometry.

(The same protocol is useful for all the different activation marker techniques, changing mitogens, and incubation time depending on each case).

4. Evaluation of cellular function

The assessment of lymphocyte function typically refers to the study of T cell function through cell proliferation in response to polyclonal nonspecific and specific stimuli; however, this concept is more extensive and refers to any method that evaluates any of the various effectors or regulatory aspects of subset of lymphocytes [33]. As we have seen previously, these studies are of particular importance when the functional impact of a mutation is unknown; in such cases, flow cytometry provides a number of different immune function assessment techniques, including evaluation of specific immune cell line functions, cell activation, proliferation, and cytokine production.

4.1 Lymphocyte proliferation assay

Lymphocyte phenotype studies should be accompanied by laboratory tests assessing the functional immunity of T cells when a primary immunodeficiency is

suspected [34]. Lymphocyte proliferation assay is used to determine lymphocyte activation and their ability of carry out cell-mediated immune response. Ligand binding and transmembrane signal transduction result in cell activation, proliferation of B and T cells which ultimately leads to clonal expansion. Flow cytometry techniques are used to evaluate *in vitro* different cell types proliferation, including the use of fluorescent follow-up cell tracking intracellular dyes [e.g., succinimidyl ester of carboxyfluorescein diacetate (CFSE)] which are capable of covalent binding to cytoplasmic structures of the cell and remain there for a long period of time, being redistributed into daughter cells by each cell division resulting in a 50% decrease in fluorescence intensity after activation for each cell division cycle [35]. Almost all lymphocytes can be stimulated to proliferate non-specifically by stimulating them *in vitro* with polyclonal or mitogenic stimulants such as phytohemagglutinin (PHA), concanavaline A, mitogen pokeweed (PWM), or anti-CD3 antibodies alone or in combination with anti-CD28 or IL-2. Generally, these trials involve at least 72-h or 6–7 days of culture depending on the stimulation (**Figure 7**). The use of flow cytometry has gradually replaced "gold standard" methods such as lymphocyte proliferation by incorporating [3H] thymidine into the DNA of dividing cells and measured using a liquid scintillation counter, which is proportional to the amount of cell proliferation. In terms of complexity, methods based on flow cytometry are considerably simpler to perform, and the undoubted advantage of this method is that the cells grow without any negative influence during the entire culture period, compared to thymidine. However, the interpretation is less objective and time-consuming, thus requiring experienced staff [36].

Cell proliferation induced by an activation signal is a critical parameter in the diagnosis of patients with a possible T-cell defect, including infants with abnormal T-cell receptor excision circles (TRECs) during newborn screening test. In these cases, the T-cell response to polyclonal stimuli is usually less than 10% of the lower limit of the reference value. However, there are patients with hypomorphic mutations that allow some degree of T cell response, often <30% of the lower limit of the reference value. Some laboratories use more than one dose of mitogen or mitogens, although this is not done routinely and is generally not necessary to detect significant defects in T cell immunity. There have been rare cases where mitogen-induced

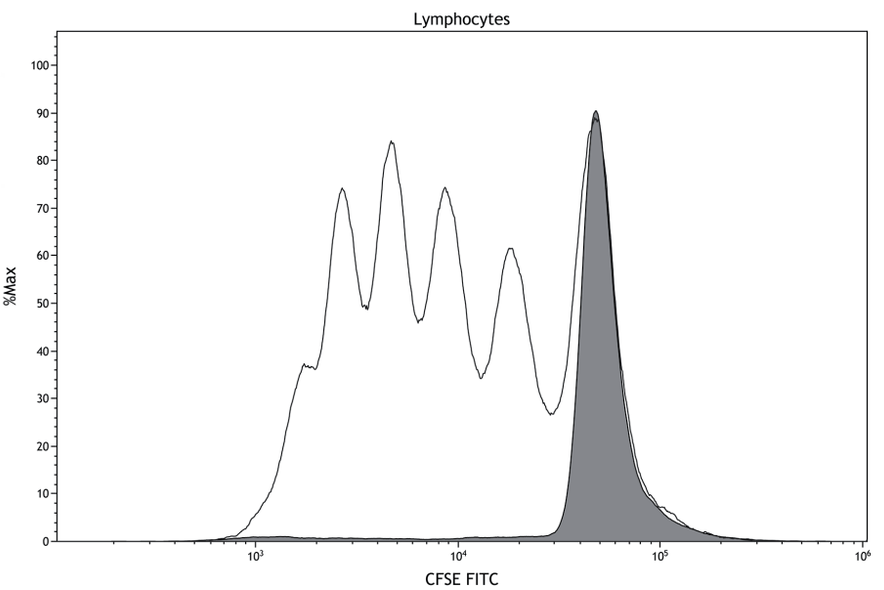

Figure 7.
Histograms of lymphocyte proliferation analysis after stimulation with PMA plus ionomicin. Lymphocytes staining with CFSE lose fluorescence after cell division. The histogram on the right shows the result of a patient, with no cell division shown compared to the result of a healthy donor on the histogram on the left.

proliferation was found to be abnormal, but further evaluation with a combination of agents that directly activate T cells [forbol 12-miristate 13-acetate (PMA) and ionomycin] showed that the proliferative capacity of T cells was normal, but the signaling apparatus (i.e., T[TCR]-CD3 cell antigen receptor-complex) was dysfunctional [7]. Obviously, one of the advantages of using cytometry to evaluate lymphocyte proliferation is the elimination of radioactive reagents. It also allows distinguishing proliferating cell subpopulations, but evaluation of the results is more subjective.

Lymphocyte proliferation assay protocol (CFSE):

1. Add 1 μL of carboxyfluorescein succinimidyl ester (CFSE) in 1 mL of PBMCs (all the PBMCs of your sample).

2. Manually gently stir the sample during 5 min.

3. Add 10 mL of Roswell Park Memorial Institute (RPMI) medium and centrifugate at 1500 rpm (324 g) during 5 min.

4. Count and adjust cells at 10^6 cells/mL.

5. Acquire 50 μL of the sample by flow cytometry just to make sure your sample has been correctly stained with the CFSE.

6. Dispense 100 μL of the PBMCs at 10^6 cells/mL in a 96 well plate.

7. Leave one well unstimulated (with RPMI) and add 100 μL of mitogens of interest in each different well [phorbol myristate acetate (PMA), ionomycin, phytohaemagglutinin (PHA), muromonab-CD3 (OKT3), interleukin-2 (IL-2), pokeweed mitogen (PWM), and concanavalin A (ConA)].

8. Incubate during 6 days at 37°C and in the dark.

9. Resuspend cell button and transfer it to fluorescence-activated cell sorter (FACS) tubes.

10. Stain with appropriate mix of antibodies of interest.

11. Incubate for 20 min at room temperature (RT) and in the dark.

12. Wash with 2 mL of phosphate buffered saline (PBS).

13. Discard the PBS after 5 min of centrifugation at 1500 rpm.

14. Add 100 μL of PBS and the sample is ready to be acquired by flow cytometry.

4.2 Functional analysis studying STAT phosphorylation

Signal transducer and activator of transcription (STAT) control cellular activation processes after cell activation through cytokines. All STAT molecules are phosphorylated by receptor-associated kinases, that causes activation, by forming homo- or heterodimers and finally translocate to nucleus to function as transcription factors [37]. The study of the different STATs by flow cytometry is of great interest not only of STAT deficiencies (STAT1, STAT3 or STAT5 deficiencies) but also to study the proper

functioning of a signaling pathway in particular. When studying STAT phosphorylation, three essential factors must be taken into account: (A) the cell lineage in which STAT phosphorylation will be studied. In the study of PID patients, it is a limitation since it is usual that not all cell lineages are present; (B) choosing the correct recombinant cytokine is crucial as cytokine receptors are expressed differently in distinct lymphocyte lineages; (C) and selecting the appropriate antibodies against the STAT phosphorylation sites [30]. These studies can also be performed by WB, which is a very sensitive but more time-consuming technique and delays results [38].

STAT phosphorylation staining protocol:

1. Stain 100 μL of heparin whole blood with appropriate mix of antibodies of interest.
2. Incubate for 20 min at RT and in the dark.
3. Wash with 2 mL of PBS.
4. Discard the PBS after 5 min of centrifugation at 1500 rpm.
5. Add 100 μL of appropriate stimuli.
6. Incubate for 20 min at 37°C and in the dark.
7. Lyse the red cells using 1 mL of lyse and fix solution.
8. Incubate for 15 min at 37°C and in the dark.
9. Discard the lyse and fix solution after 5 min of centrifugation at 1500 revolutions per minute (rpm).
10. Wash with 1 mL of PBS with fetal bovine serum (FBS) at 10%.
11. Discard the PBS + FBS 10% after 5 min of centrifugation at 1500 rpm.
12. Add 500 μL of permeabilization buffer and incubate 30 min on ice or 4°C and in the dark.
13. Add the appropriate volume of intracellular anti-phosphorylated-STAT antibody.
14. Incubate for 30 min at RT and in the dark.
15. Wash with 1 mL of PBS with FBS at 10%.
16. Discard the PBS + FBS 10% after 5 min of centrifugation at 1500 rpm.
17. Add 100 μL of PBS + FBS 10% and the sample is ready to be acquired by flow cytometry.

(The same protocol is useful for the assay of different STATs. Mitogens/cytokines and antibodies may change depending on the STAT you are studying. Note that different commercial kits can be used to assess phospho-proteins, so the protocol can be slightly different in each case).

4.2.1 STAT1 phosphorylation for the diagnosis of chronic mucocutaneous candidiasis

STAT1 is a transcription factor which in humans can be activated by several ligands such as interferon alpha (IFNα), interferon gamma (IFNγ), epidermal growth factor (EGF), platelet derived growth factor (PDGF) or interleukin 6 (IL-6). Mutations in the STAT1 molecule can be gain of function (GOF) or loss of function (LOF). Both of them can cause PID with different phenotypes and symptoms [39].

STAT1 GOF dominant mutations were first discovered in patients with chronic mucocutaneous candidiasis (CMC). This disease typically shows symptoms such as persistent infections of the skin, oral/genital mucosa and nails caused mainly by *Candida albicans*. CMC may very often result from primary immunodeficiency. To assess the presence of a STAT1 GOF defect, isolated PBMCs are stimulated *in vitro* with INFγ. In order to know if there is a gain of function, we perform different experimental conditions, such as unstimulated, stimulated with INFγ, and with INFγ plus *Staurosporine*. *Staurosporine* is an inhibitor for Ca^{2+}/calmodulin-dependent protein kinase II inhibiting STAT1 phosphorylation [40]. The use of *Staurosporine* is justified to clarify the difference between control subjects (STAT1 WT alleles) and the CMC patients (STAT1 GOF alleles). STAT1 phosphorylation is assessed in CD14+ monocytes [41]. In a healthy donor, we can observe that STAT1 phosphorylation is reduced with the addition of Staurosporine, while in a patient with STAT1 GOF, phosphorylation is maintained despite the use of Staurosporine (**Figure 8**) [42].

Regarding **STAT1 LOF autosomic recessive mutations**, there are two genetic deficiencies that compromise the response to interferons type I and III. On the one hand, there is autosomal recessive deficiency (RA), partial or even complete STAT1. These types of defects are diagnosed in patients with an increase in intracellular or viral bacterial infections, with impaired responses to IFN α, β, γ, and IL27. In complete recessive form, there is a very low response to anti-viral and antimycotical treatments. On the other hand, partial STAT1 deficiency can also be autosomal dominant (AD); phenotypically causes impaired IFNγ responses and patients suffer with selective intracellular bacterial diseases such as Mendelian susceptibility to mycobacterial diseases (MSMD). To assess if there is a STAT1 LOF, isolated PBMCs

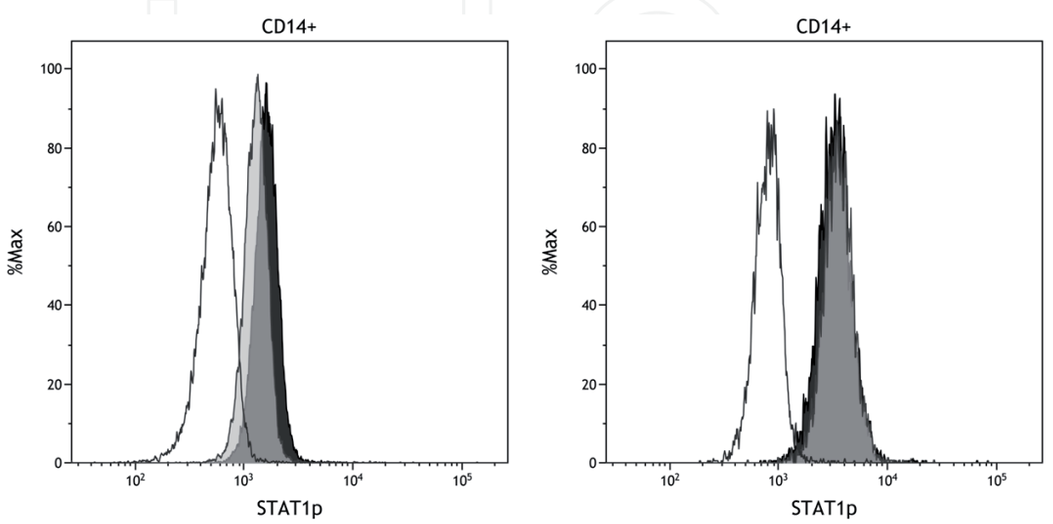

Figure 8.
The STAT1 phosphorylation and de-phosphorylation study for the diagnosis of function gain mutations (GOF) is evaluated in CD14+ monocytes. In a healthy donor (left), we can see that STAT1 phosphorylation is reduced with the addition of staurosporine (gray) while in the patient (right), phosphorylation is maintained despite the use of staurosporine (black: no staurosporine, white: control of isotypes).

are stimulated with INFγ and phosphorylation is assessed in CD14+ monocytes. In these cases, STAT1 phosphorylation in monocytes is clearly diminished comparing with a healthy donor [43].

See STAT phosphorylation staining protocol in Section 4.2.

4.2.2 STAT3 phosphorylation for the diagnosis of Hiper IgE Syndrome (HIES)

Hiper IgE Syndrome (HIES) is a PID defined as an association of atopy in a context of very high serum IgE levels, characteristic bacterial and fungal diseases, low-level clinical and biological inflammation, and various non-hematopoietic developmental manifestations. Somewhat arbitrarily, three disorders were successively put forward as the underlying cause of HIES: autosomal dominant (AD) STAT3 deficiency, the only disorder corresponding to the original definition of HIES, and autosomal recessive (AR) DOCK8 and PGM3 deficiencies, in which atopy and high serum IgE levels occur in a context of manifestations not seen in patients with typical HIES. Indeed, these three disorders disrupt different molecular pathways, affect different cell types, and underlie different clinical phenotypes [44].

Phenotype tests discussed in previous chapters, demonstrating low number of Th17 lymphocytes, may guide in-depth study of a defect in STAT3 signaling. These are the analysis of the ability to produce IL-17 by stimulation with PMA and ionomicin, adding a Golgi Plug such as Brefeldin and subsequent intracellular staining against IL17. And the STAT3 phosphorylation test in lymphocytes. Whole blood is stimulated with IL-6 and/or IL-21 and intracellular staining is performed to evaluate STAT3 phosphorylation. Different conditions are needed, such as: not stimulated, stimulated, and both with STAT3 antibodies or with an isotype control to have internal controls. Phosphorylation is clearly decreased in a patient with STAT3 deficiency compared to a healthy control [45, 46].

See STAT phosphorylation staining protocol in Section 4.2.

4.2.3 STAT5 phosphorylation for the diagnosis of severe combined immunodeficiency (SCID)

X-linked severe combined immunodeficiency (X-SCID) is an inherited disorder of the immune system caused by inborn errors in the gene IL2RG, which encodes interleukin IL-2 receptor common γ chain. The γc is a transmembrane protein shared by different cytokine receptors IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. In the absence of an effective γc function, early lymphoid progenitor cells are not able to respond properly to these cytokine signals leading low or absence of T and NK cells and hypogammaglobulinemia despite a normal B cell count [47]. T−B+NK− phenotype is characteristic for X-SCID and JAK3 deficiencies. T−B+NK+ SCID include IL-7Rα, CD3δ, CD3ε, and CD3ζ deficiencies. X-SCID represents close to half of all SCID patients. Patients with X-SCID are prone to severe, recurrent, and persistent infections caused usually in their first 6 months of life. The disease is lethal within the first 2 years of life, unless they undergo hematopoietic stem cell transplantation (HSCT) or gene therapy [48, 49].

In addition to the more deleterious mutations, which cause the most severe phenotype of the disease, some other several patients with a milder form of combined immunodeficiency, often referred to as "leaky" or "hypomorphic" SCID. Most of these cases of "leaky" SCIDs are due to hypomorphic mutations. Due to their poorly defined clinical and immunological phenotype, patients with the atypical forms are diagnosed later in childhood or even in adulthood [50].

A study of expression of the common gamma chain (CD132), expressed broadly on T and B lymphocytes, NK cells, monocytes, and granulocytes, can be performed by flow cytometry [3]. However, as mentioned above, the presence of protein does not rule out a possible functional defect. It is therefore advisable to evaluate the signaling of the IL2-IL2RG-JAK3-STAT5 pathway, studying the phosphorylation of STAT5. For this purpose, whole blood is stimulated with IL-2 and STAT5 phosphorylation is evaluated in lymphocytes. It should be noted that a patient with X-SCID will not have T lymphocytes or NK cells, therefore the test should be performed on B lymphocytes and it is important to consider the inclusion of specific B cell lineage markers. STAT5 phosphorylation is clearly reduced in patients with a common gamma chain deficiency compared to a healthy control [39]. This assay can be performed under suspicion of mutations in the following genes: IL2RG, JAK3, STAT5 or IL-7Rα.

See STAT phosphorylation staining protocol in Section 4.2.

4.3 NK cell cytotoxicity and degranulation assay evaluation

Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening condition characterized by persistent macrophage/lymphocyte activation and a multisystemic hyperinflammatory syndrome. The clinical diagnosis of HLH is challenging and requires a composite of several findings. According to the updated criteria of the Histiocyte Society [51], the diagnosis is currently established by fulfilling 5 of 8 clinical and laboratory criteria, or the finding of a genetic defect consistent with HLH. Defects associated with HLH are generally associated with abnormal NK cell function. The characterization of these patients requires genetic and functional studies, among the latter are the study of cytotoxic capacity and degranulation.

In *cytotoxicity assays*, we measure the functional capacity of natural killer cells. In this assay, PBMCs or purified NK cell are co-incubated at different ratios with a target HLA-null cell lines (usually K562) known to be sensitive to NK cell-mediated cytotoxicity [52]. After an incubation period, dead target cells are identified by nucleic acid staining, which specifically penetrates the dead cells and is measured by flow cytometry [53]. Chromium-51 (51Cr) release assays are also commonly used for accurate and precise quantification of cytotoxicity. The main disadvantages of this method are the use of radioactivity, which can be hazardous to health, and are impractical and costly due to the short half-life of 51Cr and to the need for radiological safety training and authorization requirements [54].

Cytotoxicity assay protocol:

1. Count and adjust patient PBMCs at 10^6 cells/mL concentration in a final volume of 5 mL of RPMI.

2. Count and adjust K562 cells at 2×10^5 cells/mL concentration in a final volume of 5 mL of RPMI.

3. Incubate both cell types separately at 37°C over night.

4. Count and adjust patient PBMCs at 2.5×10^6 cells/mL.

5. Count and adjust K562 cells at 0.2×10^6 cells/mL.

6. Make a serial dilution of the patient's PBMCs in FACS tubes, adding the same concentration of K562 cell line in each tube.

7. 5 μL of propidium iodide (PI) are added at each tube to identificate death cells through flow cytometry.

8. PBMcs mixed with K562 at different dilutions are incubated during 4 h at 37°C and in the dark.

9. Samples are ready to be acquired by flow cytometry.

Other lineage-specific function tests include evaluation of *NK cell degranulation* by evaluation of CD107a (LAMP1) surface expression, which is normally expressed in cytoplasmic granules, following stimulation with a target cell line (K562) or PMA plus ionomycin. This assay complements the classical NK cell cytotoxicity assay and is particularly useful in screening for the diagnosis of familial HLH. Specifically, the lack of surface expression of CD107a after incubation with K562 target cells is consistent with syntaxin-11 (STX11), uncoordinated mammals 13.4 (UNC13D), syntaxin-2 binding protein (STXBP2), or Rab27A protein defects [55].

NK cell degranulation assay protocol:

1. Count and adjust patient PBMCs at 10^6 cells/mL concentration.

2. Count and adjust K562 cells at 2×10^5 cells/mL concentration.

3. Dispense 100 μL of PBMCs at 10^6 cells/mL in a 96-well plate.

4. Leave three wells unstimulated by adding 100 μL of RPMI in each.

5. Add 100 μL of IL-2 to other three wells.

6. Incubate at 37°C over night.

7. Resuspend cell button and transfer it to FACS tubes.

8. Add 100 μL of RPMI into a tube unstimulated and into a IL-2 tube.

9. Add 100 μL of PHA into a tube unstimulated and into a IL-2 tube.

10. Add 100 μL of K26 (10^6 cells/mL) into a tube unstimulated and into a IL-2 tube.

11. Add 5 μL of anti-CD107a into all FACS tubes.

12. Incubate all FACS tubes during 3 h at 37°C.

13. Add the appropriate mix of surface antibodies of interest.

14. Incubate for 20 min at RT and in the dark.

15. Wash with 2 mL of PBS.

16. Discard the PBS after 5 min of centrifugation at 1500 rpm.

17. Add 100 μL of PBS and the sample is ready to be acquired by flow cytometry.

4.4 Detection of intracellular IFN-γ expression in activated T lymphocytes

The use of intracellular cytokine staining technique has become extremely useful in recent years to elucidate defects in intracellular signaling as causes of different IDPs. One of the applications of this technique is the characterization of the different subpopulations of T helper (Th) lymphocytes. The strategy of characterization of these subpopulations is to identify T helper 1 lymphocytes (Th1) as cells producing INFγ, T helper 2 lymphocytes (Th2) as those producing IL-4 and IL-13 and T helper 17 lymphocytes (Th17) as those producing IL-17a and IL-17f [1].

In patients with STAT3 or DOCK8 deficiency, a decrease in IL-17 producing T cells (Th17) has been demonstrated. Th17 cells play a role in autoimmunity and defense of extracellular pathogens (fungi, bacteria, and parasites). Therefore, the absence of IL-17 production after stimulation of PBMCs in these patients allows identifying a functional defect. However, mutational genetic analysis is necessary for a definitive diagnosis [56].

Moreover, this technique can be used to determine the production of other cytokines such as INFγ in front MSMD suspicion [57, 58] or the production of TNF-α in IRAK4 suspicion in defects of the innate immunity [3].

In summary, the intracellular cytokine staining technique consists of the stimulation of heparinized whole blood with PMA and ionomycin or other stimuli. After the appropriate incubation time for each cytokine, intracellular and superficial staining is performed. There are three aspects to consider: (A) the results will be compared with the cytokine production of a healthy control; (B) the incubation time with the stimulus can be modified depending on the cytokine we want to study. In case we want to study more than one, we will have to find an ideal incubation time for each of them, that is, leave time for all of them to be synthesized, without degrading any of them; (C) it is a key to know the cell subpopulation producing the cytokine of interest, as we can add surface markers that will facilitate analysis and interpretation (**Figure 9**).

Intracellular cytokine staining protocol:

1. Stimulate 500 μL of heparin whole blood with PMA and ionomycin.

2. Add 1 μL of GolgiPlug.

3. Incubate for 18 h at 37°C.

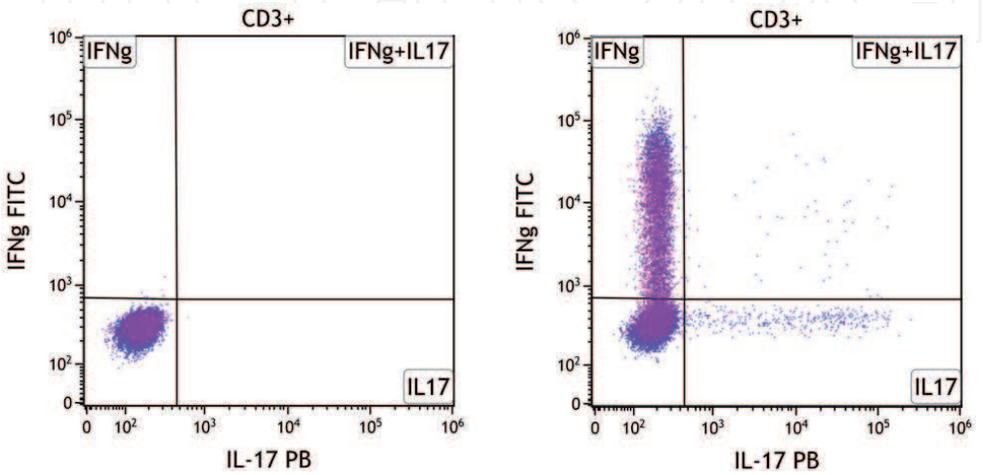

Figure 9.
Intracellular production of IFNg and IL2 cytokines. In the dotplots, cytokine production is observed after 18 h stimulation in a healthy donor (left) and a patient with an immune deficit (right).

4. Aliquot 200 μL of sample into different FACS tubes.

5. Wash with 2 mL of PBS.

6. Discard the PBS after 5 min of centrifugation at 1500 rpm (324 g).

7. Fix with the appropriate volume of fixative reagent.

8. Incubate for 15 min at RT and in the dark.

9. Add the appropriate volume of permeabilizing reagent.

10. Add the extracellular antibodies mix.

11. Add the intracellular antibodies mix.

12. Incubate for 20 min at RT and in the dark.

13. Add the appropriate volume of wash buffer.

14. Discard the wash buffer after 5 min of centrifugation at 1500 rpm.

15. Add 100 μL of PBS and the sample is ready to be acquired by flow cytometry.

(This protocol may change slightly depending on the commercial kit used).

5. Conclusions

Flow cytometry is an accessible technique in clinical laboratories; the implementation of this technology has progressed together with the significant improvements in instrumentation and the availability of a wide variety of monoclonal reagents. While the main clinical indications for immunophenotyping typically have been the quantification of CD4 T lymphocytes in HIV infection, the study of the extended immunophenotype and functional studies by flow cytometry offers nowadays advanced diagnostic approaches. Previously used techniques for the study of cell function (^{3}H-thymidine incorporation, ^{51}Cr-release assay, etc.) using radioactivity have been gradually replaced by flow cytometry. Flow cytometry can provide rapid and accurate identification of expanded lymphocyte subpopulations, which play an important role in the evaluation and understanding of complex disorders such as primary immunodeficiencies and other immunomediated diseases.

The application of flow cytometry techniques in the evaluation of PID assesses lymphocyte proliferation, intracellular changes associated with activation, cytokine production, and biological effects associated with immune defects and functional immune abnormalities.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature. 1996;**383**(6603): 787-793

[2] Olbrich P, de Felipe B, Delgado-Pecellin C, Rodero R, Rojas P, Aguayo J, et al. A first pilot study on the neonatal screening of primary immunodeficiencies in Spain: TRECS and KRECS identify severe T- and B-cell lymphopenia. Anales de Pediatría. 2014;**81**(5):310-317

[3] Kanegane H, Hoshino A, Okano T, Yasumi T, Wada T, Takada H, et al. Flow cytometry-based diagnosis of primary immunodeficiency diseases. Allergology International. 2018;**67**(1):43-54

[4] Hsieh EWY, Hernandez JD. Novel tools for primary immunodeficiency diagnosis: Making a case for deep profiling. Current Opinion in Allergy and Clinical Immunology. 2016;**16**(6):549-556

[5] Carr EJ, Dooley J, Garcia-Perez JE, Lagou V, Lee JC, Wouters C, et al. The cellular composition of the human immune system is shaped by age and cohabitation. Nature Immunology. 2016;**17**(4):461-468

[6] Garcia-Prat M, Álvarez-Sierra D, Aguiló-Cucurull A, Salgado-Perandrés S, Briongos-Sebastian S, Franco-Jarava C, et al. Extended immunophenotyping reference values in a healthy pediatric population. Cytometry Part B: Clinical Cytometry. 2018;**96**(3):223-233

[7] Fleisher TA, Madkaikar M, Rosenzweig SD. Application of flow cytometry in the evaluation of primary immunodeficiencies. Indian Journal of Pediatrics. 2016;**83**(5):444-449

[8] Corneo B, Moshous D, Güngör T, Wulffraat N, Philippet P, Le Deist FL, et al. Identical mutations in RAG1 or RAG2 genes leading to defective V(D)J recombinase activity can cause either T-B-severe combined immune deficiency or Omenn syndrome. Blood. 2001;**97**(9):2772-2776

[9] Fink PJ. The biology of recent thymic emigrants. Annual Review of Immunology. 2013;**31**(1):31-50

[10] Sallusto F. Heterogeneity of human CD4(+) T cells against microbes. Annual Review of Immunology. 2016;**34**(1):317-334

[11] van den Broek T, Borghans JAM, van Wijk F. The full spectrum of human naive T cells. Nature Reviews. Immunology. 2018;**18**(6):363-373

[12] Farber DL, Yudanin NA, Restifo NP. Human memory T cells: Generation, compartmentalization and homeostasis. Nature Reviews. Immunology. 2014;**14**(1):24-35

[13] Azizi G, Rezaei N, Kiaee F, Tavakolinia N, Yazdani R, Mirshafiey A, et al. T-cell abnormalities in common variable immunodeficiency. Journal of Investigational Allergology & Clinical Immunology. 2016;**26**(4):233-243

[14] Dieli-Crimi R, Martínez-Gallo M, Franco-Jarava C, Antolin M, Blasco L, Paramonov I, et al. Th1-skewed profile and excessive production of proinflammatory cytokines in a NFKB1-deficient patient with CVID and severe gastrointestinal manifestations. Clinical Immunology. 2018;**195**:49-58

[15] Bacchetta R, Barzaghi F, Roncarolo M-G. From IPEX syndrome to FOXP3 mutation: A lesson on immune dysregulation. Annals of the New York Academy of Sciences. 2018;**1417**(1):5-22

[16] Warnatz K, Schlesier M. Flowcytometric phenotyping of common

variable immunodeficiency. Cytometry Part B: Clinical Cytometry. 2008;**74B**(5):261-271

[17] Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: Defining subgroups in common variable immunodeficiency. Blood. 2008;**111**(1):77-85

[18] Avery DT, Kane A, Nguyen T, Lau A, Nguyen A, Lenthall H, et al. Germline-activating mutations in PIK3CD compromise B cell development and function. The Journal of Experimental Medicine. 2018;**215**(8):2073-2095

[19] Orange JS. Natural killer cell deficiency. The Journal of Allergy and Clinical Immunology. 2013;**132**(3):515-525

[20] Orange JS. Unraveling human natural killer cell deficiency. Journal of Clinical Investigation. 2012;**122**(3):798-801

[21] Nichols BA, Bainton DF, Farquhar MG. Differentiation of monocytes. Origin, nature, and fate of their azurophil granules. The Journal of Cell Biology. 1971;**50**(2):498-515

[22] Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: Their role in infection and inflammation. Journal of Leukocyte Biology. 2007;**81**(3):584-592

[23] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;**392**(6673):245-252

[24] Spinner MA, Sanchez LA, Hsu AP, Shaw PA, Zerbe CS, Calvo KR, et al. GATA2 deficiency: A protean disorder of hematopoiesis, lymphatics, and immunity. Blood. 2014;**123**(6):809-821

[25] Marcondes NA, Fernandes FB, Spindler BM, Faulhaber GAM. Flow cytometry assessment of intracellular BTK expression. Cytometry. Part B, Clinical Cytometry. 2018;**94**(5):568

[26] Gifford CE, Weingartner E, Villanueva J, Johnson J, Zhang K, Filipovich AH, et al. Clinical flow cytometric screening of SAP and XIAP expression accurately identifies patients with SH2D1A and XIAP/BIRC4 mutations. Cytometry. Part B, Clinical Cytometry. 2014;**86**(4):263-271

[27] Kogawa K, Lee SM, Villanueva J, Marmer D, Sumegi J, Filipovich AH. Perforin expression in cytotoxic lymphocytes from patients with hemophagocytic lymphohistiocytosis and their family members. Blood. 2002;**99**(1):61-66

[28] Demaret J, Saison J, Venet F, Malcus C, Poitevin-Later F, Lepape A, et al. Assessment of a novel flow cytometry technique of one-step intracellular staining: Example of FOXP3 in clinical samples. Cytometry. Part B, Clinical Cytometry. 2013;**84**(3):187-193

[29] Sava F, Toldi G, Treszl A, Hajdú J, Harmath Á, Tulassay T, et al. Expression of lymphocyte activation markers of preterm neonates is associated with perinatal complications. BMC Immunology. 2016;**17**(1):19

[30] Lim LC, Fiordalisi MN, Mantell JL, Schmitz JL, Folds JD. A whole-blood assay for qualitative and semiquantitative measurements of CD69 surface expression on CD4 and CD8 T lymphocytes using flow cytometry. Clinical and Diagnostic Laboratory Immunology. 1998;**5**(3):392-398

[31] O'Gorman MR. Measurement of CD40 ligand (CD154) expression on resting and in vitro-activated T cells. Current Protocols in Cytometry. 2001;Chapter 6:Unit 6.7

[32] Snyder JT, Shen J, Azmi H, Hou J, Fowler DH, Ragheb JA. Direct

inhibition of CD40L expression can contribute to the clinical efficacy of daclizumab independently of its effects on cell division and Th1/Th2 cytokine production. Blood. 2007;**109**(12):5399-5406

[33] Albert-Vega C, Tawfik DM, Trouillet-Assant S, Vachot L, Mallet F, Textoris J. Immune functional assays, from custom to standardized tests for precision medicine. Frontiers in Immunology. 2018;**16**(9):2367

[34] Oliveira JB, Fleisher TA. Laboratory evaluation of primary immunodeficiencies. The Journal of Allergy and Clinical Immunology. 2010;**125**(Suppl 2):S297-S305

[35] Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. Journal of Immunological Methods. 1994;**171**(1):131-137

[36] Lašťovička J, Rataj M, Bartůňková J. Assessment of lymphocyte proliferation for diagnostic purpose: Comparison of CFSE staining, Ki-67 expression and 3H-thymidine incorporation. Human Immunology. 2016;**77**(12):1215-1222

[37] Casanova J-L, Holland SM, Notarangelo LD. Inborn errors of human JAKs and STATs. Immunity. 2012;**36**(4):515-528

[38] Bitar M, Boldt A, Binder S, Borte M, Kentouche K, Borte S, et al. Flow cytometric measurement of STAT1 and STAT3 phosphorylation in CD4 + and CD8 + T cells—Clinical applications in primary immunodeficiency diagnostics. The Journal of Allergy and Clinical Immunology. 2017;**140**(5): 1439-1441.e9

[39] Lorenzini T, Dotta L, Giacomelli M, Vairo D, Badolato R. STAT mutations as program switchers: Turning primary immunodeficiencies into autoimmune diseases. Journal of Leukocyte Biology. 2017;**101**(1):29-38

[40] Yanagihara N, Tachikawa E, Izumi F, Yasugawa S, Yamamoto H, Miyamoto E. Staurosporine: An effective inhibitor for Ca^{2+}/calmodulin-dependent protein kinase II. Journal of Neurochemistry. 1991;**56**(1):294-298

[41] Mizoguchi Y, Tsumura M, Okada S, Hirata O, Minegishi S, Imai K, et al. Simple diagnosis of STAT1 gain-of-function alleles in patients with chronic mucocutaneous candidiasis. Journal of Leukocyte Biology. 2014;**95**(4):667-676

[42] Depner M, Fuchs S, Raabe J, Frede N, Glocker C, Doffinger R, et al. The extended clinical phenotype of 26 patients with chronic mucocutaneous candidiasis due to gain-of-function mutations in STAT1. Journal of Clinical Immunology. 2016;**36**(1):73-84

[43] Chapgier A, Wynn RF, Jouanguy E, Filipe-Santos O, Zhang S, Feinberg J, et al. Human complete Stat-1 deficiency is associated with defective type I and II IFN responses in vitro but immunity to some low virulence viruses in vivo. Journal of Immunology. 2006;**176**(8):5078-5083

[44] Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, et al. STAT3 mutations in the hyper-IgE syndrome. The New England Journal of Medicine. 2007;**357**(16):1608-1619

[45] Meshaal SS, El Hawary RE, Eldash A, Grimbacher B, Camacho-Ordonez N, Abd Elaziz DS, et al. Diagnosis of DOCK8 deficiency using flow cytometry biomarkers: An Egyptian center experience. Clinical Immunology. 2018;**195**:36-44

[46] Renner ED, Rylaarsdam S, Anover-Sombke S, Rack AL, Reichenbach J, Carey JC, et al. Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell numbers, and variably defective

STAT3 phosphorylation in hyper-IgE syndrome. The Journal of Allergy and Clinical Immunology. 2008;**122**(1):181-187

[47] Leonard WJ, Noguchi M, Russell SM, McBride OW. The molecular basis of X-linked severe combined immunodeficiency: The role of the interleukin-2 receptor gamma chain as a common gamma chain, gamma c. Immunological Reviews. 1994;**138**:61-86

[48] Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, et al. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. Cell. 1993;**73**(1):147-157

[49] Kovanen PE, Leonard WJ. Cytokines and immunodeficiency diseases: Critical roles of the gamma(c)-dependent cytokines interleukins 2, 4, 7, 9, 15, and 21, and their signaling pathways. Immunological Reviews. 2004;**202**(1):67-83

[50] Mou W, He J, Chen X, Zhang H, Ren X, Wu X, et al. A novel deletion mutation in IL2RG gene results in X-linked severe combined immunodeficiency with an atypical phenotype. Immunogenetics. 2017;**69**(1):29-38

[51] Janka GE, Lehmberg K. Hemophagocytic syndromes—An update. Blood Reviews. 2014;**28**(4): 135-142

[52] Lisovsky I, Isitman G, Bruneau J, Bernard NF. Functional analysis of NK cell subsets activated by 721.221 and K562 HLA-null cells. Journal of Leukocyte Biology. 2015;**97**(4):761-767

[53] Kandarian F, Sunga GM, Arango-Saenz D, Rossetti M. A flow cytometry-based cytotoxicity assay for the assessment of human NK cell activity. Journal of Visualized Experiments. 2017;**9**(126):56191-56199

[54] Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: Comparisons to a 4 h 51Cr-release assay. Journal of Immunological Methods. 2007;**325**(1-2):51-66

[55] Rubin TS, Zhang K, Gifford C, Lane A, Choo S, Bleesing JJ, et al. Perforin and CD107a testing is superior to NK cell function testing for screening patients for genetic HLH. Blood. 2017;**129**(22):2993-2999

[56] Sandquist I, Kolls J. Update on regulation and effector functions of Th17 cells. F1000Research. 2018;**7**:205

[57] Esteve-Solé A, Sologuren I, Martínez-Saavedra MT, Deyà-Martínez À, Oleaga-Quintas C, Martinez-Barricarte R, et al. Laboratory evaluation of the IFN-γ circuit for the molecular diagnosis of Mendelian susceptibility to mycobacterial disease. Critical Reviews in Clinical Laboratory Sciences. 2018;**55**(3):184-204

[58] Feinberg J, Fieschi C, Doffinger R, Feinberg M, Leclerc T, Boisson-Dupuis S, et al. Bacillus Calmette Guerin triggers the IL-12/IFN-gamma axis by an IRAK-4- and NEMO-dependent, non-cognate interaction between monocytes, NK, and T lymphocytes. European Journal of Immunology. 2004;**34**(11):3276-3284