Chapter

Flow Cytometric Approach in the Diagnosis of Primary Immunodeficiencies

Sevil Oskay Halacli

Abstract

Primary Immunodeficiencies (PIDs) compose of a large spectrum of diseases characterized by abrogated or dysregulated functions of innate and adaptive immune system components that cause susceptibility to recurrent infections, auto-immunity, neoplasia/malignancy and dysfunction of organs and skeletal system. PIDs are also evaluated as molecular diseases due to the mutations in one or more genes. That affects transcripts and protein expressions as well as their functions. Today, 430 different genes are known to have various functional effects which are related to 403 different PIDs. Analyzing the effects of the mutations on relevant protein expression and function is significant to diagnose and the follow-up of the PIDs. Application of flow cytometry for analyzing protein expression levels and functions in immune cells as well as investigating the cellular functions tender a rapid, quantitative and reliable approach to identify and to prove the genetic background of PIDs. Therefore, the use of flow cytometry aids to have a large spectrum of data from gene to function and from function to clinical relevance in the first-step and differantial diagnosis of PIDs.

Keywords: Primary immunodeficiency, flow cytometry, molecular diagnosis, immunophenotyping, PBMC culture, functional assays, intracellular staining, PI3K pathway analysis- flow, CFSE cell proliferation

1. Introduction

Primary immunodeficiencies (PIDs) are rare and heterogenous genetic diseases of the immune system. According to updated IUIS (International Union of Immunological Societies) classification in 2019, there is a large spectrum of PIDs including 403 different diseases caused by mutations in 430 genes categorized 10 different subclasses with these topics: Severe combined immunodeficiencies (SCIDs), combined immunodeficiencies (CIDs) less profound than SCID, CIDs with associated or syndromic features and predominantly antibody deficiencies including common variable immunodeficiency (CVID), immune dysregulation, phagocyte system defects, innate immune defects, auto-inflammation, complement deficiencies, bone marrow abnormalities and phenocopies of PIDs. Each disease has unique laboratory and clinical manifestations. Decreased or increased immune cell counts, unbalanced immune cell plasticity, decreased or increased immunoglobulin levels and complement factors, dysregulated functions of immune cells due to

abrogated intracellular molecular functions cause developing clinical manifestations of PIDs [1]. Use of flow cytometry in these laboratory investigations is a significant approach that offers a quantitative, reliable and rapid results. Evaluation of these laboratory findings helps to clinicians for proper diagnose of PIDs [2, 3].

2. Analysis of inflammatory and regulatory cell profiles in PIDs

Immune dysregulation with autoimmunity is observed in many PIDs such as LRBA, CTLA4, STAT3 GOF, PIK3CD deficiencies as well as IPEX syndrome caused by loss or dysfunctional FOXP3 expression [4–18]. Disrupted T helper cell plasticity is pointed out as a prominent feature of the autoimmunity in PIDs. Deregulated numbers and functions of Treg cells are observed in most of the patients with IPEX or IPEX-like (such as in patients with LRBA deficiency) [6, 7, 19–21]. Decreased Treg cell numbers or loss of Treg cell functions are related to severe form of autoimmunities in PIDs. In contrast, deregulated inflammatory cell numbers/ratios and the inflammatory cytokines produced by inflammatory cells are observed as autoimmune manifestations of PIDs such as LRBA and STAT3 LOF deficiencies. In LRBA deficiency, increased number of circulating T folicular helper (Tfh) is associated with autoimmune manifestations of the disease [5]. Moreover, decreased Th17 cell numbers are related to inflammatory response to Candida infections observed in patients with LOF mutations in STAT3 deficiency [22–24].

In these cases, the first attempt is to analyze regulatory and inflammatory cell ratios in the clinical immunology laboratory to clarify the cellular background of autoimmunity.

2.1 Analysis of Treg cells in PIDs

Treg cells are unique subset of T helper cells through its equilibrating functions on immune response to self and foreign antigens. Tregs suppress inflammatory T cell function and proliferation, therefore it plays critical roles to prevent autoimmune disorders. In PIDs with autoimmunity, impaired functions of Treg cells in parallel with decreased number of Treg cells are observed. IPEX is a well-known syndrome affecting Treg cell development due to mutations of FOXP3 which is a main transcription factor in the development of Treg cells. In patients with IPEX syndrome, loss of circulating and tissue associated Treg cells are thought to cause the multi-organ autoimmune manifestations [6, 20, 21]. Patients with CD25 (IL-2Rα) deficiency have IPEX-like phenotype as well as in patients with LRBA deficiency. Decreased Treg ratio is a significant laboratory characteristics in these PIDs [7, 25]. In patients with AIRE deficiency which is related to Autoimmune Poly Endocrinopathy, Candidiasis and Ectodermal Dystrophy (APECED) syndrome, decreased Treg cell ratio and function are associated with the occurrence of the disease [26].

Investigating Treg cell ratio by flow cytometry provides an important insight to understand autoimmunity from the benchside to bedside.

Below, it was described the Treg staining protocol and the gating strategy for human peripheral blood Treg cells (**Figure 1**).

2.1.1 Treg staining protocol

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 4 ml of whole blood in tube with EDTA.

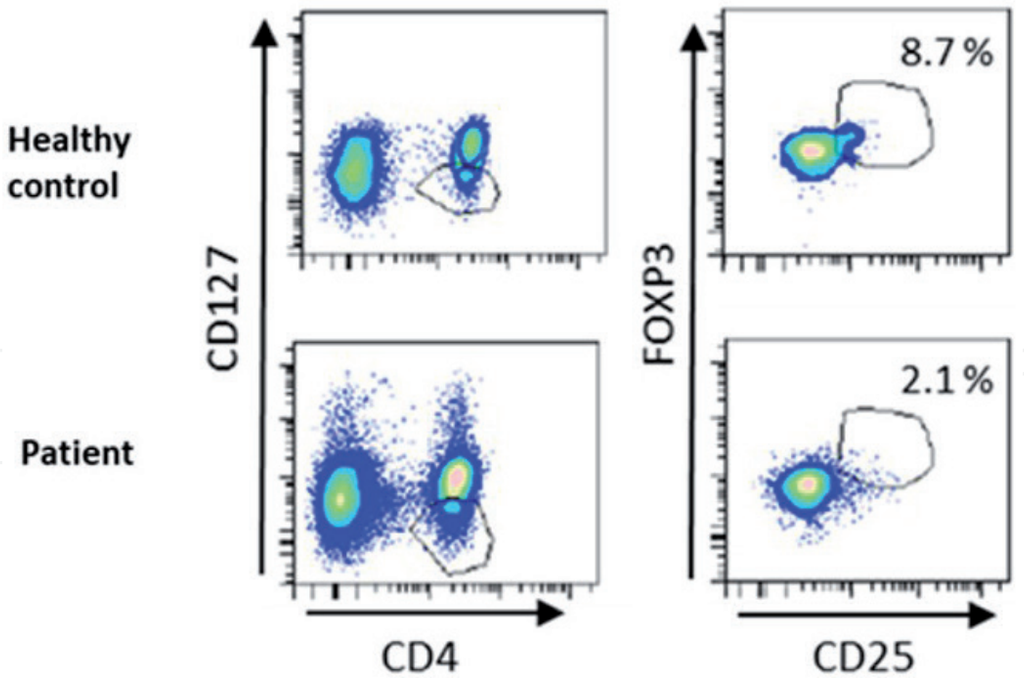

Figure 1.
Representative image of CD4+ CD127^{lo}CD25^{hi} FOXP3+ Treg cells in peripheral blood of healthy control and a patient.

- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant
- Add appropriate volume of PBS and add 100 ul cell to flow cytometer tubes
- Add appropriate volume of CD4, CD127 and CD25 antibodies and incubate at room temperature and dark conditions for 20 min
- Following incubation wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Fix the cells with a fixation buffer for 10–20 min
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Treat with the permeabilization buffer for 10–30 min
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Add FOXP3 antibody for 30 min at room temperature and dark conditions
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Add 300 ul PBS, vortex and analyze in flow cytometer

2.2 Analysis of circulating Tfh and TH17 cells in PIDs

Tfh cells are specialized Th cell subset which plays important role in B cell differentiation in lymph nodes, in producing high affinity antibodies and the

development of memory cells. Therefore, Tfh provides help germinal center (GC) formation and selection of plasma cells [27–30]. Tfh cells have unique molecules that are expressed in cell surface and have special functions such as CXCR5. CXCR5 is a chemokine receptor and provides migration of Tfh cells to GC zone. Besides, Tfh expresses B Cell Lymphoma (BCL-6) and (Inducible T Cell Costimulator) ICOS or CD278 on their surfaces. Increased Tfh cell numbers in peripheral blood are investigated as an inflammatory marker of some PIDs such as LRBA deficiency [5].

Th17 cells are also a subset of helper T cells which are responsible for producing IL-17, a pro-inflammatory cytokine recruiting neutrophils to infection site to combat infection [22, 23, 31, 32]. IL-6 expression and STAT3 activation are required for the differentiation of Th17 cells from CD4+ T lymphocytes. Therefore in STAT3 deficiency caused by autosomal dominant loss of function mutations of STAT3 gene, decreased number of circulating Th17 cells are associated with susceptibility to Candida infections in STAT3 LOF deficiency which is a type of Autosomal Dominant- hyper IgE Syndrome (AD-HIES) [24].

Detection of Tfh and Th17 cell ratios in the peripheral blood of the patients with designated PIDs in clinical immunology laboratory by flow cytometry using various surface and intracellular markers which are unique to circulating Tfh and Th17 cells is important step to understand the inflammatory background of the autoimmune manifestations (**Figures 2** and **3**). See the Section 2.1.1. for the staining protocol.

Below, it was demonstrated Tfh and Th17 gating strategy.

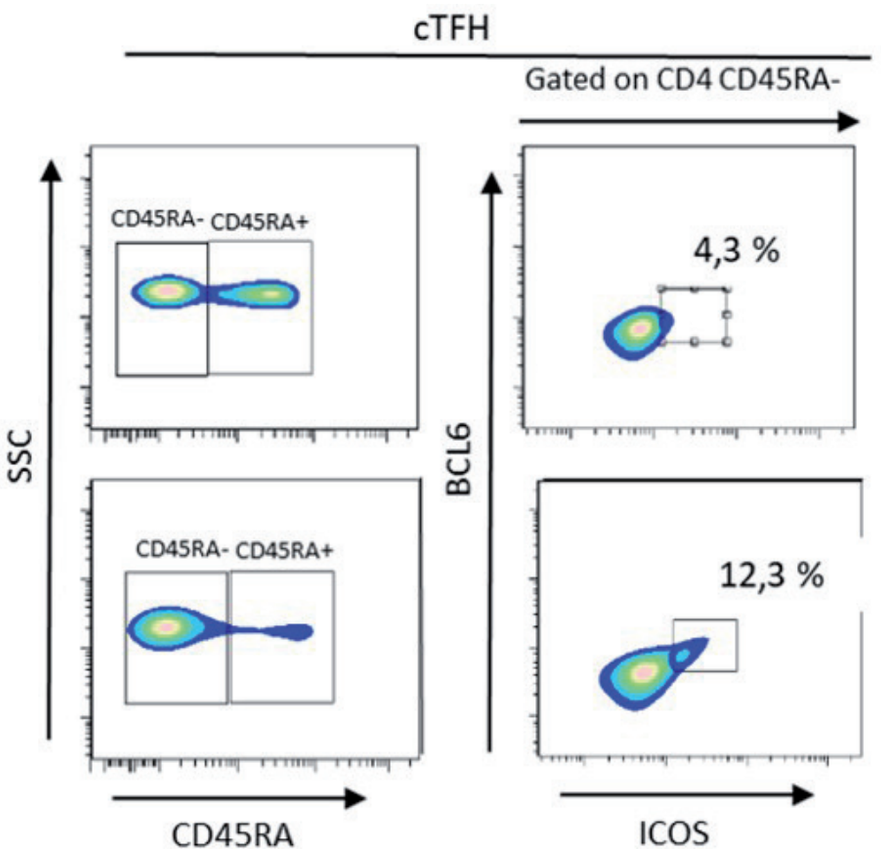

Figure 2.
Analysis of cTfh cells in a healthy control (top) and a patient with PID (below). In the patient, increased ratio of cTfh is observed.

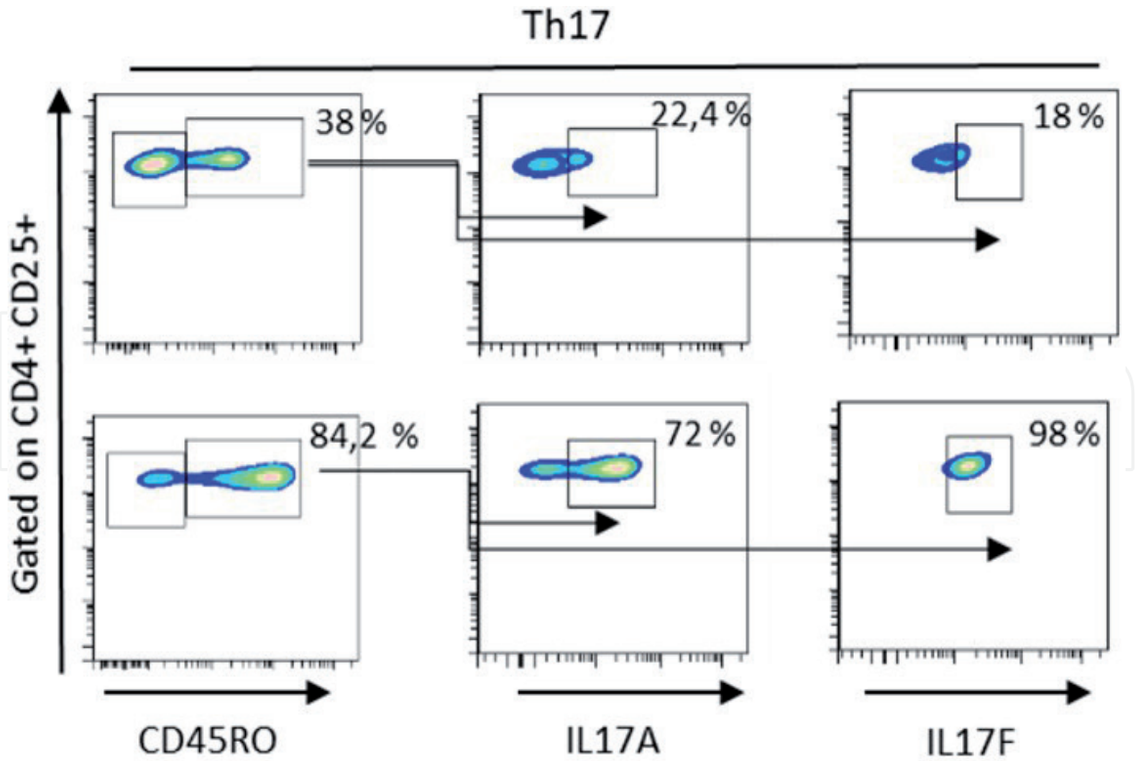

Figure 3.
Th17 gating strategy. Increased ratio of Th17 cells expressing IL17A and IL17F are observed in a patient (below) compared to healthy control (top).

3. Analysis of surface molecules in PIDs

3.1 Evaluation of molecules which are constitutively expressed on cell surface

In the diagnosis of suspicious patients for PID, flow cytometry is frequently applied to detect specific molecules which are expressed on specific subset of immune cells in clinical immunology research laboratory [2, 3]. It is used for immunophenotyping as well as in the detection of specific protein expression in cells. In the evaluation of constitutively expressed proteins on cell surface, activation with specific stimulus is not required. CD40 and CD55 deficiencies are the examples which are described in detail in Section 3.1.1. and 3.1.2 for the surface protein expression analysis in PIDs.

In the staining of surface proteins, fixation and permeabilization steps are not needed. Therefore staining protocol is easier and faster than intracellular staining of the proteins which is described in Section 4. Following staining protocol is used to detect surface protein expressions in PIDs:

- Add 100 ul of whole blood to flow cytometer tube.
- Add appropriate volume of specific antibodies to detect specific proteins and incubate at room temperature and dark conditions for 20–30 min.
- Lyse the erythrocytes using appropriate volume of lysis buffer and incubate for 10–15 min at room temperature and dark conditions.
- Centrifuge at 500 g for 5 min and discard the supernatant

- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Add 300 ul PBS, vortex and analyze at flow cytometer

3.1.1 CD40 deficiency in hyper IgM syndrome

CD40 is a costimulatory molecule which is expressed on antigen presenting cells such as B cells, macrophages and dendritic cells. CD40 interacts with CD40L on T cells in GC zones and is activated in the maturation of B cells and isotype switching [33, 34]. Similar to CD40L deficiency, CD40 deficiency is investigated for suspicious Hyper IgM syndromes. Decreased or unfunctional CD40 expression on B lymphocyte as well as CD40L expression defects on T cells in suspicious patients for Hyper IgM syndrome is related to disease occurrence [35, 36]. See the Section 3.1. for the staining protocol.

3.1.2 CD55 expression in CHAPLE syndrome

Decay-accelerating factor (DAF) or CD55 is an inhibitor molecule of complement system and it is related to various diseases and a recently described PID which is named as (CD55 deficiency with hyperactivation of complement, angiopathic thrombosis, and PLE) CHAPLE syndrome. Because CD55 acts as an inhibitor of complement system, low or loss of expressions due to mutations in its encoding gene, complement system is more active in patients than healthy individuals [37–39] (see the Section 3.1. for the staining protocol).

3.2 Analysis of the expression of induced surface proteins in PIDs

3.2.1 CD40L expression in T lymphocytes in hyper IgM syndrome

CD40L, also known as CD154, is expressed on T cells and responsible for the interaction with CD40 which is expressed on antigen presenting cells such as B cells. CD40L is a member of TNF-receptor superfamily and its interaction with CD40 on B cells is associated with Ig class switching, affinity maturation and GC formation. In most of the patients with CD40L deficiency, loss or decreased CD40L protein expression on T cells are associated with increased levels of soluble IgM levels and decreased IgG and IgA levels are investigated [35, 36]. Expression of CD40L protein on T cell surface is very low and increased by activation using Phorbol Myristate Acetate (PMA) and ionomycin inducing transcriptional activity of NFAT and AP-1 transcription factors in T cells following T cell receptor stimulation. Following 3 hours of activation of PBMCs, CD69 which is an early activation marker and CD40L expression are detected on T cell surface (**Figure 4**). Staining protocol of CD40L and CD69 on CD3+ CD8- T cells are as in below:

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA.
- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with serum free media.

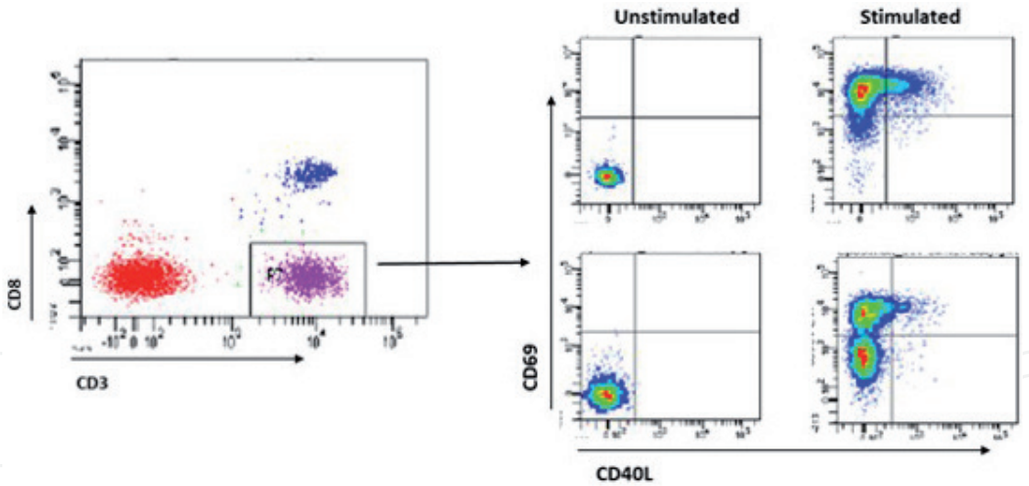

Figure 4.
Gating strategy for CD40L and CD69 expression on CD3+ CD8- T cells in unstimulated and stimulated samples from a healthy control (top) and a patient (below).

- Prepare two flasks for each sample to analyze unstimulated and stimulated samples
- Put the appropriate number of cells to culture flask. Add 1 ug/ml PMA and 500 ng/ml ionomycin to the stimulated culture flask
- Following 3 hours incubation in humidified incubator, wash the cells with PBS and centrifuge at 300 g for 5 min and discard the supernatant
- Resuspend the cells with 1 ml PBS and collect 100 ul of cell to a fresh flow cytometer tubes
- Add CD3, CD8, CD69 and CD40L antibodies at the appropriate concentrations
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Add 300 ul PBS, vortex and analyze at flow cytometer

3.2.2 CD70 expression

CD27/CD70 signaling pathway is significant for the immune response to Epstein–Barr virus (EBV) infections. CD27 is expressed on T lymphocytes as well as B lymphocytes and whereas its ligand, CD70, is limited to induced T and B lymphocytes and dendritic cells. CD27-CD70 signaling is responsible for T cell survival, Treg activity, B cell differentiation and proliferation. Due to CD27-CD70 partnership in immune response against to EBV, similar clinical characteristics are monitored in patients with CD27 and CD70 deficiencies [40–42]. EBV-associated lymphoproliferative disorder, lymphoma, hypogammaglobulinemia and autoimmune manifestations are generalized clinical symptoms in both deficiencies [41, 42]. Therefore, analyzing of CD27 and CD70 proteins in PBMCs using flow cytometry due to its rapid and quantitative analysis guide to clinicians as a first step molecular diagnosis of patients with these clinical manifestations before sequencing. **Figure 5** shows the gating strategy for CD70 staining. Staining protocol for CD27 is as in Section 3.1.

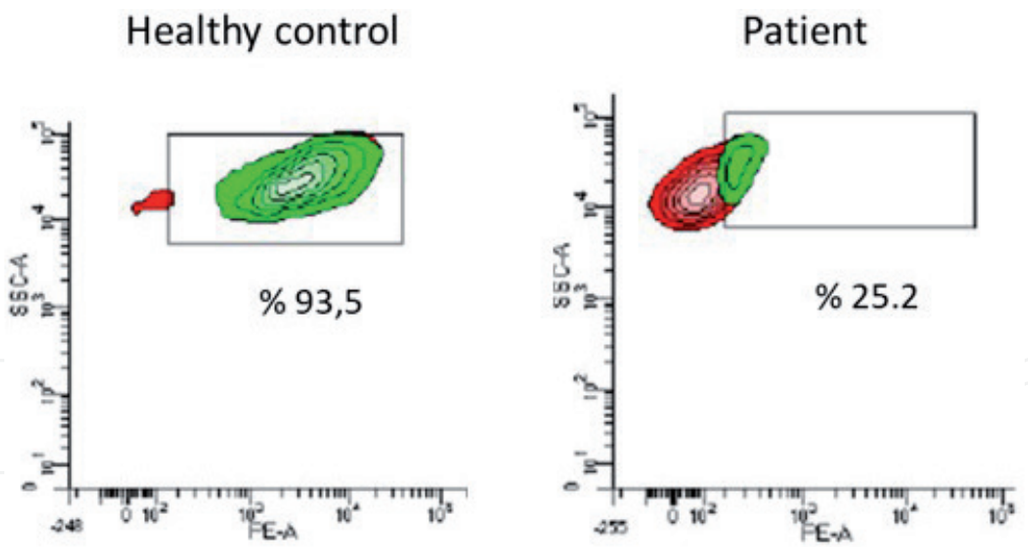

Figure 5.
A representative image of CD70 expression on CD19+ B lymphocyte in a healthy control and a patient.

CD70 activation and staining protocol is as below:

3.2.2.1 Activation of surface expression of CD70 and staining for flow cytometric analysis

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA
- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with serum free media
- Prepare two flasks for each sample to analyze unstimulated and stimulated samples
- Put the appropriate number of cells to culture flask and add 2,5 ug/ml phyto-hemagglutinin (PHA) in the completed culture media
- Incubate the cells in humidified incubator for 72 hours
- After 72 hours add appropriate volume of IL-2 to the cells
- At the day of 8, wash the cells with PBS
- Centrifuge at 500 g for 5 min and discard the supernatant
- Add appropriate volume of CD70 antibody and incubate for 30 min at room temperature
- Wash the cells with PBS and Centrifuge at 500 g for 5 min and discard the supernatant
- Resuspend the cells with 300 ul PBS and analyze at flow cytometer.

3.2.3 CTLA4 (CD152)

Cytotoxic T lymphocyte Antigen-4 (CTLA4) is an inhibitor ligand of T lymphocytes which bind to CD80/CD86 which is found on antigen presenting cells with higher affinity than a costimulator molecule CD28 [8–10]. CTLA4 ceases signaling axes in T lymphocytes due to its ITIM motifs in the intracytoplasmic domain. Therefore CTLA4 blocks T cell proliferation and act important function in homeostasis and peripheral tolerance. CTLA4 is constitutively expressed on T lymphocytes and it is expressed on cell surface only after stimulation via TCR and Ca+/Calcineurin pathway *in vitro*. In patients with autosomal dominant mutation of CTLA4, lymphadenopathy/splenomegaly, hypogammaglobulinemia, cytopenia and organ specific autoimmunity are observed. This disease is also called "haploinsufficiency with autoimmune infiltration (CHAI) disease" and characterized by unfunctional or loss of CTLA4 expression on T lymphocytes [8–10]. Using flow cytometric approach, suspicious patients with CHAI disease may be investigated for molecular diagnosis before sequencing. **Figure 6** demonstrates the gating strategy for CTLA4 expression in healthy control and a patient with PID. Flow cytometry protocol for CTLA4 activation and staining are below:

3.2.3.1 Staining protocol of CTLA4 in activated PBMCs

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA

- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with serum free media

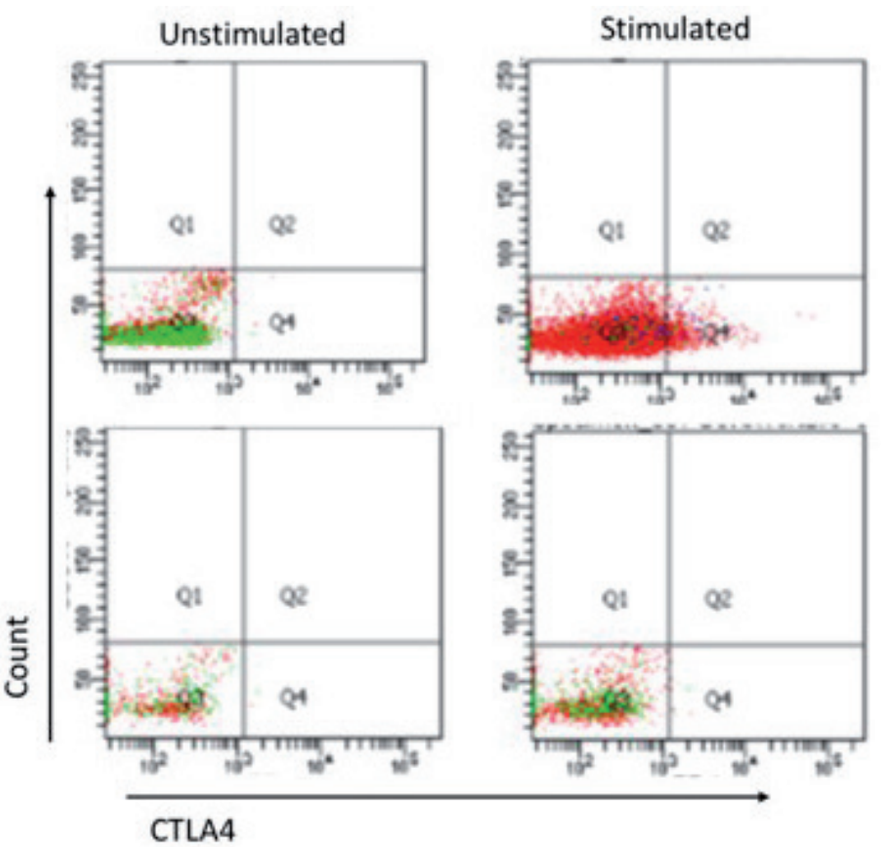

Figure 6.
A representative image of CTLA4 expression in unstimulated and stimulated PBMC samples obtained from in a healthy control (top) and a patient (below). Decreased CTLA4 expression was observed in the patient compared to the healthy control.

- Prepare two flasks for each sample to analyze unstimulated and stimulated samples
- Put the appropriate number of cells to culture flask and add 5 ug/ml (PHA) in the completed culture media
- Incubate the cells overnight in humidified incubator
- Wash the cells with PBS
- Centrifuge at 500 g for 5 min and discard the supernatant
- Add appropriate volume of CTLA4 antibody and incubate for 30 min at room temperature
- Wash the cells with PBS and centrifuge at 500 g for 5 min and discard the supernatant
- Resuspend the cells with 300 ul PBS and analyze at flow cytometer

4. Analysis of intracellular molecules in PIDs

4.1 Single protein evaluation in related cell population by flow cytometry

The following protocol is applied to the patients who have suggestive clinical history related to LRBA, STK4, DOCK8 and BTK deficiencies before and after sequencing to evaluate the alteration of designated protein expressions.

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA
- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with PBS
- Add appropriate volume of PBS and add 100 ul cell to flow cytometer tubes
- Add appropriate volume of antibodies related to cells which are interested for 30 min
- Following incubation wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Fix the cells with a fixation buffer for 10–20 min
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Treat with the permeabilization buffer for 10–30 min
- Wash with PBS, centrifuge at 500 g for 5 min and discard the supernatant
- Incubate with related antibody for 30 min

- Wash the cells with PBS and centrifuge at 500 g for 5 min and discard the supernatant

- Resuspend the cells with 300 ul PBS and analyze at flow cytometer

4.1.1 LRBA deficiency

(Lipopolysaccharide responsive beige-like anchor protein) LRBA plays important roles in vesicle trafficking and receptor recycling. LRBA is responsible for CTLA4 trafficking from vesicular compartments to the cell membrane. In patients with LRBA mutations, an autosomal recessive form of combined immunodeficiency arises and this deficiency is associated with hypogammaglobulinemia, recurrent respiratory infections, multiple autoimmune manifestations and frequently susceptibility to inflammatory bowel disease and malignity in some cases [4, 6, 7, 43–45]. See the Section 4.1. for the staining protocol. **Figure 7** shows a representative image of LRBA expression in LRBA deficient patient and a healthy control.

4.1.2 STK4 (MST1) deficiency

STK4 (serine–threonine protein kinase 4), also known as MST1 (Macrophage Stimulating 1), was first found in *Drosophila* as a member of the Hippo pathway, which regulates proliferation and cell survival. Human STK4 is principally discovered as a constitutively expressed kinase, structurally homologous to the *Drosophila* Hippo, and plays roles in vital biologic processes such as morphogenesis, proliferation, apoptosis, and stress response [46–49]. STK4 deficiency was first defined in 2012 by 3 separate groups as causing a novel autosomal recessive CID, which is characterized by a profoundly decreased level of CD4+ T cells with the concomitant tendency to recurrent viral and bacterial infections and mucocutaneous candidiasis [46, 49]. Mutations in STK4 gene cause the lack of protein expression or severely reduced level of protein expression [50] (**Figure 8**). See the Section 4.1. for the staining protocol.

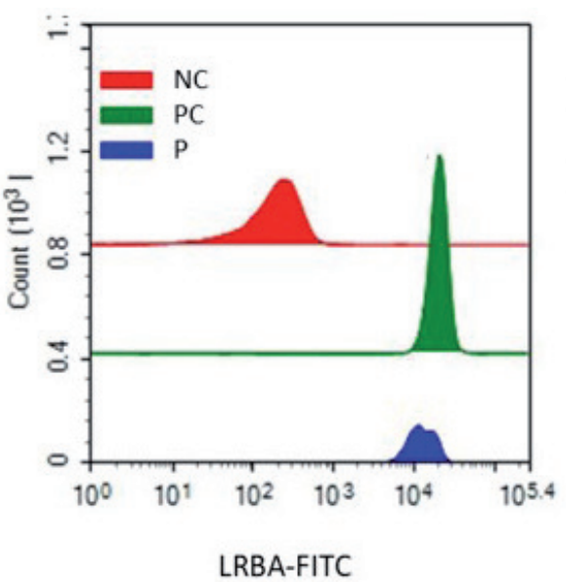

Figure 7.
A representative image of LRBA expression in a negative control (NC or isotype control), positive or healthy control (PC) and a patient (P). Decreased LRBA expression was observed in the patient compared the PC.

4.1.3 DOCK8 deficiency

DOCK8 is a member of DOCK-C family and is responsible for activation of GTPases such as CDC42 and RAC. Therefore it transmit the signals from the membrane to intracellular compartment of cells and involves the cytoskeletal rearrangement of the cells. Decreased expression or total loss of DOCK8 protein due to bi-allelic mutations of DOCK8 gene cause Autosomal-Recessive Hyper-IgE Syndrome (AR-HIES) which is associated with eosinophilia and elevated IgE levels in the effected patients [51–53] (**Figure 9**). See the Section 4.1. for the staining protocol.

4.1.4 BTK deficiency in XLA

BTK is a member of Tec family of non-receptor tyrosine kinases and plays a role in the transmission of the signals from the membrane into the cell. BTK localizes

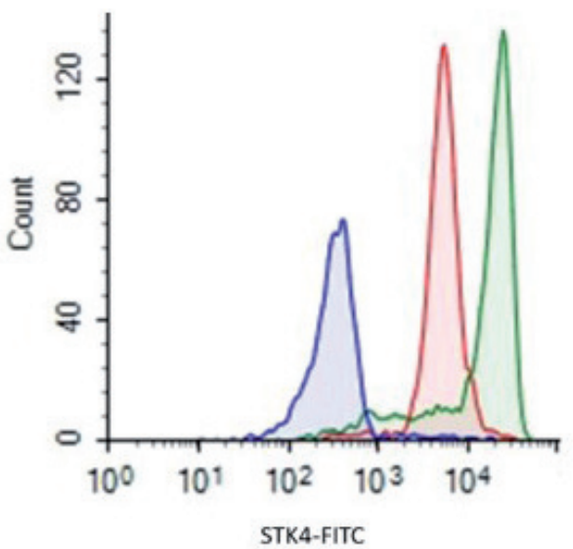

Figure 8.
A representative image of STK4 expression in isotype control (blue), healthy control (green) and the patient (red). Decreased STK4 expression was observed in the patient compared to the healthy control [50].

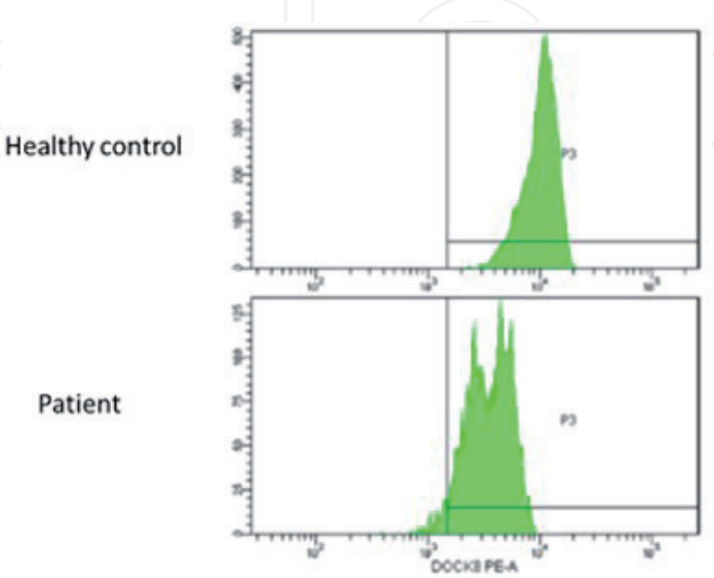

Figure 9.
A representative image of DOCK8 expression in healthy control (top) and the patient (below). Decreased DOCK8 expression was observed in the patient compared to the healthy control.

next to BCR in B cells, therefore it is important for B cell development. In mutations of BTK which is present on X-chromosome cause X-linked agammaglobulinemia in patients who suffered from recurrent bacterial infections due to low or nearly undetectable immunoglobulins and B lymphocytes [54]. Lymphocyte phenotyping is frequently used to diagnose the diseases in patients with suspicious clinical findings and BTK expression is analyzed for molecular diagnosis underlying the XLA. **Figure 10** demonstrates the BTK expression in a patients' and a healthy controls' samples. See the Section 4.1. for the staining protocol.

4.2 Pathway characterization in PIDs

4.2.1 PI3K pathway characterization

Activated phosphoinositide-3 kinase-δ syndrome (APDS) also known as p110δ-activating mutation causing senescent T cells, lymphadenopathy and immunodeficiency (PASLI) occurs in patients with combined immunodeficiency due to gain of function mutations of phosphoinositide 3-kinase (PI3K) genes PIK3CD and

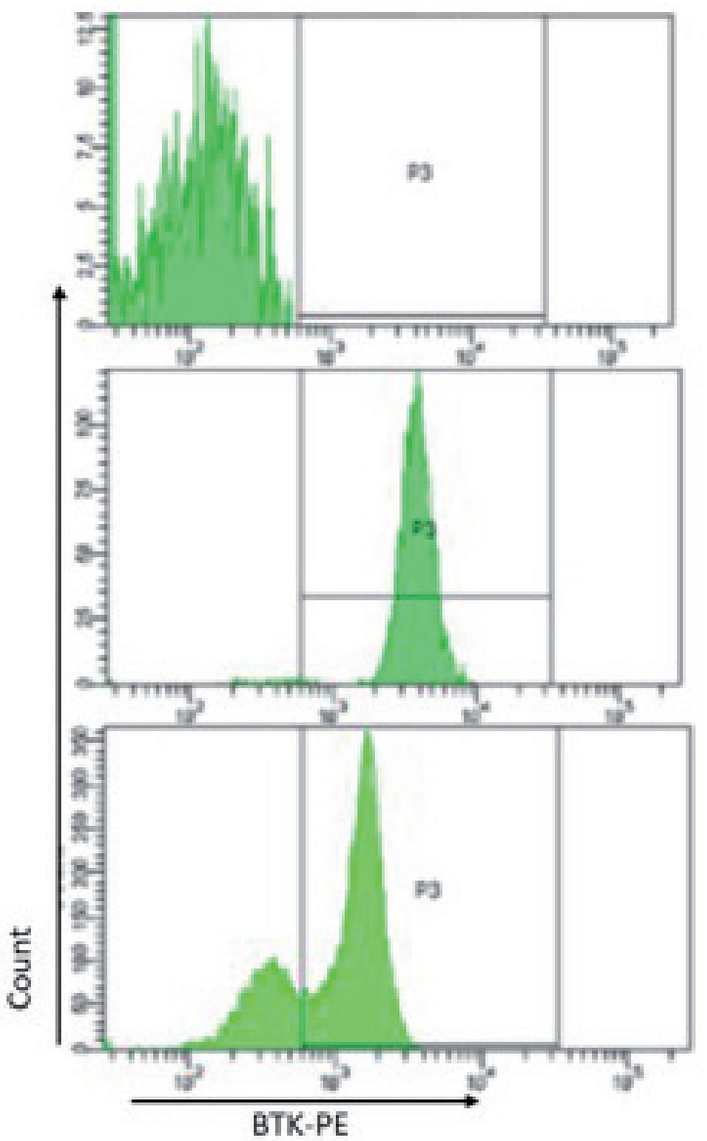

Figure 10.
BTK expression in isotype control (top) healthy control (middle) and the patient (below). BTK expression was lower in the patient than the healthy control.

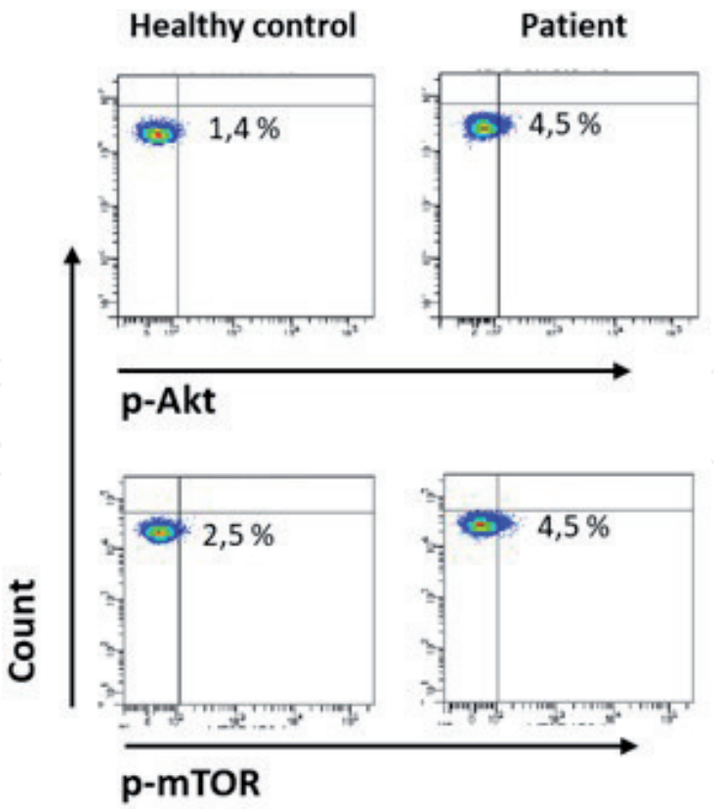

Figure 11.
Ratio of cells expressing p-Akt and p-mTOR in a patient with PIK3δ GOF deficiency and a healthy control following pathway stimulation as described in section 4.2.1.1.

PIK3R1 [14, 16–18]. Although clinical manifestations are heterogenous among the patients, recurrent and persistent infections with herpes family viruses, lymphoproliferation, immune cytopenia are observed in the majority of the patients. Investigating the pathway in patients with suggestive to APDS or PASLI, PI3K pathway analysis, downstream kinase phosphorylations with or without stimulation with specific receptors such as TCR or BCR are investigated by flow cytometry [16]. In the latter section, staining protocol of the PIK3δ, p-Akt and p-mTOR are summarized. **Figure 11** shows a representative image of p-Akt and p-mTOR expression in a patient with PIK3δ GOF deficiency and a healthy control sample.

4.2.1.1 PIK3δ and downstream pathway activation and staining protocol

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA
- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with serum free media
- Prepare two flasks for each sample to analyze unstimulated and stimulated samples
- Put the appropriate number of cells to culture flask and add an appropriate receptor activating agent to induce the pathway and incubate in humidified incubator in suggested time depend on the agent used in the activation
- Centrifuge at 500 g for 5 min and discard the supernatant

- Add appropriate volume of PIK3δ, p-Akt and p-mTOR antibodies and incubate for 30 min at room temperature
- Wash the cells with PBS and centrifuge at 500 g for 5 min and discard the supernatant
- Resuspend the cells with 300 ul PBS and analyze at flow cytometer

5. Analysis of cellular functions of immune cells

5.1 Cell proliferation

Severe combined immunodeficiencies (CIDs) including T-B + NK-, T-B-NK+, T-B-NK- and T-B + NK+ and/or isolated T cell deficiencies are severe forms of PIDs due to important roles of T lymphocytes to combat directly or indirectly protein and viral antigens [55]. T lymphocytes have specific subsets to achieve their superior roles on specific antigenic determinant. Their deficiencies due to specific molecular defects affect their activation, receptor editing, functions and proliferative capacity cause critically ill disease phenotype. They need to re-regulate their receptors and proliferate to expand agent-specific clones such an army to combat during various specific-infections. Therefore detecting cell proliferation is significant for the diagnosis and/or the course of the disease. Non-radioactive cell tracking dyes such as CFSE (carboxyfluorescein succinimidyl ester) has been started to use for the assessment of cell proliferation in flow cytometry. CFSE is a non-fluorescent dye and becomes permeable through its two acetate groups and passing through the cell membrane. After entering the cells, following the separation of acetate groups via esterases, it becomes fluorescent and its permeability is decreased. Succinimidyl group of CFSE reacts with amino groups of mostly from lysine residues of intracellular molecules such as cytoskeletal proteins and forms stable covalent bonds. In

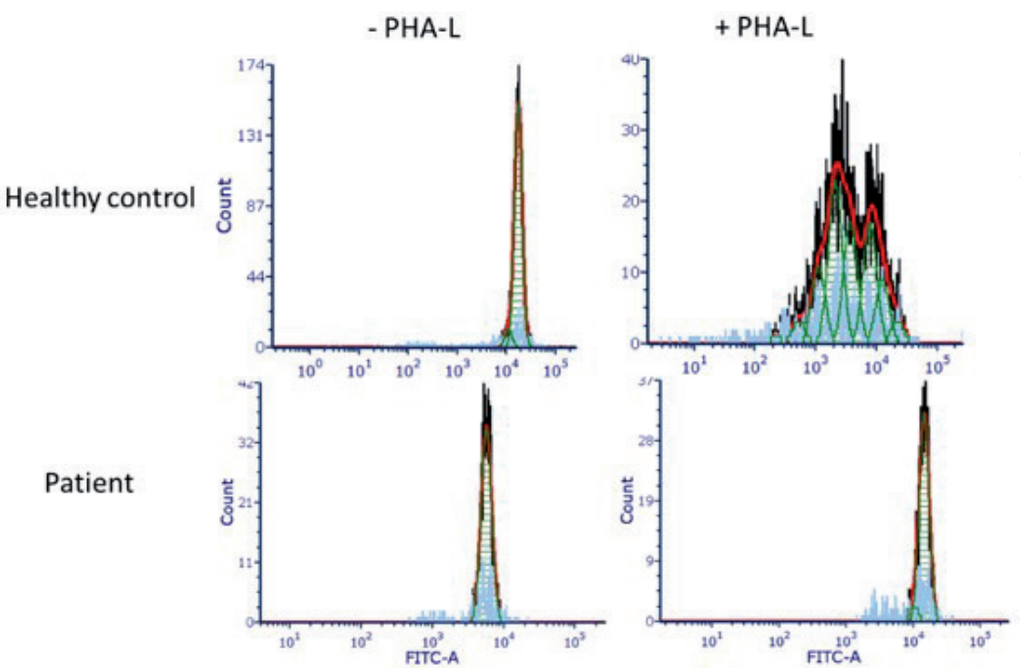

Figure 12.
Comparison of CD3+ T lymphocyte proliferation between a patient with SCID and a healthy control individual. Normal proliferation in the healthy control sample (top) and loss of CD3+ T lymphocyte proliferation in the patient with SCID (below).

each cell division its fluorescent density is decreased and this decrease in cells is evaluated in flow cytometry [56–58]. Severely affected lymphocyte proliferation in a patient with severe combined immunodeficiency is shown in **Figure 12**. See the CFSE cell staining protocol in Section 5.1.1.

5.1.1 CFSE staining protocol

- Peripheral blood mononuclear cells (PBMCs) are separated by ficoll density gradient protocol from 1 to 2 ml of whole blood in tube with EDTA
- Wash PBMCs with Phosphate Buffer Saline (PBS) buffer, centrifuge at 300 g for 5 min and discard the supernatant and resuspend the cells with serum free media
- Prepare two flasks to analyze the proliferation in unstimulated and stimulated cells
- Put the appropriate number of cells to culture flask and label them with the appropriate concentration of CFSE for 5–10 minutes in dark conditions
- Centrifuge at 500 g for 5 min and discard the supernatant for two times
- Add appropriate volume of T cell activator such as PHA (Phorbol Myristate Acetate) to stimulate the cells
- Incubate cells for 72–96 hours in humidified conditions
- Wash the cells with PBS and centrifuge at 500 g for 5 min and discard the supernatant
- Incubate with appropriate volume of anti-CD3 antibody
- Wash the cells with PBS and centrifuge at 500 g for 5 min and discard the supernatant
- Resuspend the cells with 300 ul PBS and analyze at flow cytometer.

Acknowledgements

I would like to express my sincere thanks to Prof. Ilhan Tezcan, MD, PhD and Prof. Deniz Cagdas Ayvaz, MD, PhD for their valuable supports. This study was supported by the grants with the number TSA-2018-17339 and 315S125 from Hacettepe University and TUBITAK, respectively. The authors would like to thank participants for being a part of this study.

IntechOpen

Author details

Sevil Oskay Halacli
Department of Basic Sciences of Pediatrics, Division of Pediatric Immunology, Hacettepe University, Ankara, Turkey

*Address all correspondence to: seviloskay@gmail.com

References

[1] A. Bousfiha *et al.*, "Human Inborn Errors of Immunity: 2019 Update of the IUIS Phenotypical Classification," *J. Clin. Immunol.*, vol. 40, no. 1, 2020, doi: 10.1007/s10875-020-00758-x.

[2] H. Kanegane *et al.*, "Flow cytometry-based diagnosis of primary immunodeficiency diseases," Allergology International, vol. 67, no. 1. 2018, doi: 10.1016/j.alit.2017.06.003.

[3] T. Takashima *et al.*, "Multicolor Flow Cytometry for the Diagnosis of Primary Immunodeficiency Diseases," J. Clin. Immunol., vol. 37, no. 5, 2017, doi: 10.1007/s10875-017-0405-7.

[4] G. Lopez-Herrera *et al.*, "Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity," Am. J. Hum. Genet., vol. 90, no. 6, 2012, doi: 10.1016/j.ajhg.2012.04.015.

[5] F. J. Alroqi *et al.*, "Exaggerated follicular helper T-cell responses in patients with LRBA deficiency caused by failure of CTLA4-mediated regulation," J. Allergy Clin. Immunol., vol. 141, no. 3, 2018, doi: 10.1016/j.jaci.2017.05.022.

[6] L. M. Charbonnier *et al.*, "Regulatory T-cell deficiency and immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like disorder caused by loss-of-function mutations in LRBA," J. Allergy Clin. Immunol., vol. 135, no. 1, 2015, doi: 10.1016/j.jaci.2014.10.019.

[7] S. Eren Akarcan *et al.*, "Two male siblings with a novel LRBA mutation presenting with different findings of IPEX syndrome," *JMM Case Reports*, vol. 5, no. 10, 2018, doi: 10.1099/jmmcr.0.005167.

[8] A. M. Paterson *et al.*, "Deletion of CTLA-4 on regulatory T cells during adulthood leads to resistance to autoimmunity," *J. Exp. Med.*, vol. 212, no. 10, 2015, doi: 10.1084/jem.20141030.

[9] L. R. Watson et al., "Pitfalls of immunotherapy: lessons from a patient with CTLA-4 haploinsufficiency," Allergy, Asthma Clin. Immunol., vol. 14, no. 1, 2018, doi: 10.1186/s13223-018-0272-7.

[10] K. D., R. K., and U. G., "Multilineage cytopenias in CTLA4 deficiency due to autoimmune destruction: A retrospective review," *J. Clin. Immunol.*, vol. 38, no. 3, 2018.

[11] S. Jägle *et al.*, "Distinct molecular response patterns of activating STAT3 mutations associate with penetrance of lymphoproliferation and autoimmunity," *Clin. Immunol.*, vol. 210, 2020, doi: 10.1016/j.clim.2019.108316.

[12] F. Consonni, L. Dotta, F. Todaro, D. Vairo, and R. Badolato, "Signal transducer and activator of transcription gain-of-function primary immunodeficiency/immunodysregulation disorders," *Current Opinion in Pediatrics*, vol. 29, no. 6. 2017, doi: 10.1097/MOP.0000000000000551.

[13] W. Rae *et al.*, "Autoimmunity/inflammation in a monogenic primary immunodeficiency cohort," *Clin. Transl. Immunol.*, vol. 6, no. 9, 2017, doi: 10.1038/cti.2017.38.

[14] C. L. Lucas, A. Chandra, S. Nejentsev, A. M. Condliffe, and K. Okkenhaug, "PI3Kδ and primary immunodeficiencies," Nature Reviews Immunology, vol. 16, no. 11. 2016, doi: 10.1038/nri.2016.93.

[15] S. Preite, B. Huang, J. L. Cannons, D. B. McGavern, and P. L. Schwartzberg, "PI3K orchestrates T follicular helper cell differentiation in a context dependent manner: Implications for

autoimmunity," *Frontiers in Immunology*, vol. 10, no. JAN. 2019, doi: 10.3389/fimmu.2018.03079.

[16] C. L. Lucas *et al.*, "Heterozygous splice mutation in PIK3R1 causes human immunodeficiency with lymphoproliferation due to dominant activation of PI3K," *J. Exp. Med.*, vol. 211, no. 13, 2014, doi: 10.1084/jem.20141759.

[17] E. S. J. Edwards *et al.*, "Activating PIK3CD mutations impair human cytotoxic lymphocyte differentiation and function and EBV immunity," J. Allergy Clin. Immunol., vol. 143, no. 1, 2019, doi: 10.1016/j.jaci.2018.04.030.

[18] G. L. Dornan, B. D. Siempelkamp, M. L. Jenkins, O. Vadas, C. L. Lucas, and J. E. Burke, "Conformational disruption of PI3Kδ regulation by immunodeficiency mutations in PIK3CD and PIK3R1," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 114, no. 8, 2017, doi: 10.1073/pnas.1617244114.

[19] F. Barzaghi, L. Passerini, and R. Bacchetta, "Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome: A paradigm of immunodeficiency with autoimmunity," *Front. Immunol.*, vol. 3, no. JUL, 2012, doi: 10.3389/fimmu.2012.00211.

[20] C. L. Bennett *et al.*, "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3," *Nat. Genet.*, vol. 27, no. 1, 2001, doi: 10.1038/83713.

[21] R. Bacchetta, F. Barzaghi, and M. G. Roncarolo, "From IPEX syndrome to FOXP3 mutation: A lesson on immune dysregulation," *Annals of the New York Academy of Sciences*, vol. 1417, no. 1. 2016, doi: 10.1111/nyas.13011.

[22] S. Al Khatib *et al.*, "Defects along the TH17 differentiation pathway underlie genetically distinct forms of the hyper IgE syndrome," J. Allergy Clin. Immunol., vol. 124, no. 2, 2009, doi: 10.1016/j.jaci.2009.05.004.

[23] H. D. Ochs, M. Oukka, and T. R. Torgerson, "TH17 cells and regulatory T cells in primary immunodeficiency diseases," Journal of Allergy and Clinical Immunology, vol. 123, no. 5. 2009, doi: 10.1016/j.jaci.2009.03.030.

[24] E. D. Renner *et al.*, "Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced TH17 cell numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome," *J. Allergy Clin. Immunol.*, vol. 122, no. 1, 2008, doi: 10.1016/j.jaci.2008.04.037.

[25] A. A. Caudy, S. T. Reddy, T. Chatila, J. P. Atkinson, and J. W. Verbsky, "CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes," J. Allergy Clin. Immunol., vol. 119, no. 2, 2007, doi: 10.1016/j.jaci.2006.10.007.

[26] S. M. Laakso *et al.*, "Regulatory T cell defect in APECED patients is associated with loss of naive FOXP3+ precursors and impaired activated population," J. Autoimmun., vol. 35, no. 4, 2010, doi: 10.1016/j.jaut.2010.07.008.

[27] Y. Zhu, L. Zou, and Y. C. Liu, "T follicular helper cells, T follicular regulatory cells and autoimmunity," *International Immunology*, vol. 28, no. 4. 2016, doi: 10.1093/intimm/dxv079.

[28] C. S. Ma, "Human T Follicular Helper Cells in Primary Immunodeficiency: Quality Just as Important as Quantity," Journal of Clinical Immunology, vol. 36. 2016, doi: 10.1007/s10875-016-0257-6.

[29] D. Baumjohann, D. Baumjohann, and K. M. Ansel, "Identification of T

follicular helper (Tfh) cells by flow cytometry," *Protoc. Exch.*, 2013, doi: 10.1038/protex.2013.060.

[30] C. S. Ma and T. G. Phan, "Here, there and everywhere: T follicular helper cells on the move," *Immunology*, vol. 152, no. 3. 2017, doi: 10.1111/imm.12793.

[31] A. N. Kamali *et al.*, "A role for Th1-like Th17 cells in the pathogenesis of inflammatory and autoimmune disorders," Mol. Immunol., vol. 105, 2019, doi: 10.1016/j.molimm.2018.11.015.

[32] K. Yasuda, Y. Takeuchi, and K. Hirota, "The pathogenicity of Th17 cells in autoimmune diseases," Seminars in Immunopathology, vol. 41, no. 3. 2019, doi: 10.1007/s00281-019-00733-8.

[33] E. Castigli, R. Fuleihan, N. Ramesh, E. Tsitsikov, A. Tsytsykova, and R. S. Geha, "Cd40 ligand/cd40 deficiency," *Int. Arch. Allergy Immunol.*, vol. 107, no. 1-3, 1995, doi: 10.1159/000236923.

[34] L. Murguia-Favela *et al.*, "CD40 DEFICIENCY: A UNIQUE ADULT PATIENT WITH HYPER IMMUNOGLOBULIN M SYNDROME AND NORMAL EXPRESSION OF CD40," *LymphoSign J.*, 2017, doi: 10.14785/lymphosign-2017-0004.

[35] O. Cabral-Marques *et al.*, "Expanding the clinical and genetic spectrum of human CD40L deficiency: The occurrence of paracoccidioidomycosis and other unusual infections in brazilian patients," J. Clin. Immunol., vol. 32, no. 2, 2012, doi: 10.1007/s10875-011-9623-6.

[36] X. Du *et al.*, "Clinical, genetic and immunological characteristics of 40 Chinese patients with CD40 ligand deficiency," *Scand. J. Immunol.*, vol. 90, no. 4, 2019, doi: 10.1111/sji.12798.

[37] A. Ozen, "CHAPLE syndrome uncovers the primary role of complement in a familial form of Waldmann's disease," *Immunological Reviews*, vol. 287, no. 1. 2019, doi: 10.1111/imr.12715.

[38] A. Ozen *et al.*, "CD55 Deficiency, Early-Onset Protein-Losing Enteropathy, and Thrombosis," *N. Engl. J. Med.*, vol. 377, no. 1, 2017, doi: 10.1056/nejmoa1615887.

[39] O. A. *et al.*, "Inherited CD55 deficiency in patients with early onset protein-losing enteropathy and thrombosis," *J. Pediatr. Gastroenterol. Nutr.*, vol. 64, 2017.

[40] H. Abolhassani *et al.*, "Combined immunodeficiency and Epstein-Barr virus- induced B cell malignancy in humans with inherited CD70 deficiency," *J. Exp. Med.*, vol. 214, no. 1, 2017, doi: 10.1084/jem.20160849.

[41] K. Izawa *et al.*, "Inherited CD70 deficiency in humans reveals a critical role for the CD70-CD27 pathway in immunity to Epstein-Barr virus infection," *J. Exp. Med.*, vol. 214, no. 1, 2017, doi: 10.1084/jem.20160784.

[42] S. Ghosh *et al.*, "Extended clinical and immunological phenotype and transplant outcome in CD27 and CD70 deficiency," *Blood*, 2020, doi: 10.1182/blood.2020006738.

[43] E. Lévy *et al.*, "LRBA deficiency with autoimmunity and early onset chronic erosive polyarthritis," Clin. Immunol., vol. 168, 2016, doi: 10.1016/j.clim.2016.03.006.

[44] F. Salami *et al.*, "Leishmaniasis and Autoimmunity in Patient with LPS-Responsive Beige-Like Anchor Protein (LRBA) Deficiency," *Endocrine, Metab. Immune Disord. - Drug Targets*, vol. 20, no. 3, 2019, doi: 10.2174/1871530319666190807161546.

[45] D. Cagdas *et al.*, "A Spectrum of Clinical Findings from ALPS to

CVID: Several Novel LRBA Defects," J. Clin. Immunol., 2019, doi: 10.1007/s10875-019-00677-6.

[46] S. O. Halacli *et al.*, "STK4 (MST1) deficiency in two siblings with autoimmune cytopenias: A novel mutation," Clin. Immunol., vol. 161, no. 2, 2015, doi: 10.1016/j.clim.2015.06.010.

[47] H. Abdollahpour *et al.*, "The phenotype of human STK4 deficiency," Blood, vol. 119, no. 15, 2012, doi: 10.1182/blood-2011-09-378158.

[48] B. Al-Saud *et al.*, "STK4 Deficiency in a Patient with Immune Complex Glomerulonephritis, Salt-Losing Tubulopathy, and Castleman's-Like Disease," Journal of Clinical Immunology, vol. 39, no. 8. 2019, doi: 10.1007/s10875-019-00682-9.

[49] C. Schipp *et al.*, "EBV negative lymphoma and autoimmune lymphoproliferative syndrome like phenotype extend the clinical spectrum of primary immunodeficiency caused by STK4 deficiency," *Front. Immunol.*, vol. 9, no. OCT, 2018, doi: 10.3389/fimmu.2018.02400.

[50] S. OSKAY HALACLI, D. CAGDAS, and I. TEZCAN, "Flow Cytometry is a Reliable Tool in the Diagnosis of STK4 Deficiency," *Asthma Allergy Immunol.*, 2020, doi: 10.21911/aai.518.

[51] S. Haskologlu *et al.*, "Clinical, immunological features and follow up of 20 patients with dedicator of cytokinesis 8 (DOCK8) deficiency," *Pediatr. Allergy Immunol.*, vol. 31, no. 5, 2020, doi: 10.1111/pai.13236.

[52] S. E. Aydin *et al.*, "DOCK8 Deficiency: Clinical and Immunological Phenotype and Treatment Options - a Review of 136 Patients," J. Clin. Immunol., vol. 35, no. 2, 2015, doi: 10.1007/s10875-014-0126-0.

[53] C. M. Biggs, S. Keles, and T. A. Chatila, "DOCK8 deficiency: Insights into pathophysiology, clinical features and management," Clinical Immunology, vol. 181. 2017, doi: 10.1016/j.clim.2017.06.003.

[54] S. Hashimoto *et al.*, "Identification of Bruton's tyrosine kinase (Btk) gene mutations and characterization of the derived proteins in 35 X-linked agammaglobulinemia families: A nationwide study of Btk deficiency in Japan," *Blood*, vol. 88, no. 2, 1996, doi: 10.1182/blood.v88.2.561.bloodjournal882561.

[55] R. Kumrah *et al.*, "Genetics of severe combined immunodeficiency," Genes and Diseases, vol. 7, no. 1. 2020, doi: 10.1016/j.gendis.2019.07.004.

[56] M. Koyanagi, S. Kawakabe, and Y. Arimura, "A comparative study of colorimetric cell proliferation assays in immune cells," Cytotechnology, vol. 68, no. 4, 2016, doi: 10.1007/s10616-015-9909-2.

[57] I. Terrén, A. Orrantia, J. Vitallé, O. Zenarruzabeitia, and F. Borrego, "CFSE dilution to study human T and NK cell proliferation in vitro," in *Methods in Enzymology*, vol. 631, 2020.

[58] E. Azarsiz, N. Karaca, B. Ergun, M. Durmuscan, N. Kutukculer, and G. Aksu, "In vitro T lymphocyte proliferation by carboxyfluorescein diacetate succinimidyl ester method is helpful in diagnosing and managing primary immunodeficiencies," *J. Clin. Lab. Anal.*, vol. 32, no. 1, 2018, doi: 10.1002/jcla.22216.