Flow cytometry: Surface markers and beyond

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Flow cytometry is a routinely available laboratory method to study cells in suspension from a variety of human sources. Application of this technology as a clinical laboratory method has evolved from the identification of cell-surface proteins to characterizing intracellular proteins and providing multiple different techniques to assess specific features of adaptive and innate immune function. This expanded menu of flow cytometric testing approaches has increased the utility of this platform in characterizing and diagnosing disorders of immune function. (J Allergy Clin Immunol 2019;143:528-37.)

Key words: *Immunophenotype, flow cytometry, primary immunodeficiency, immune function testing*

Abbreviations used

CD40L:	CD40 ligand
CD62L:	CD62 ligand
CGD:	Chronic granulomatous disease
CTLA4:	Cytotoxic T lymphocyte–associated protein 4
CVID:	Common variable immunodeficiency
DHR:	Dihydrorhodamine 123
DNT:	Double-negative T
DOCK8:	Dedicator of cytokinesis 8
GFP:	Green fluorescent protein
HIGM:	Hyper-IgM
LAD:	Leukocyte adhesion deficiency
LRBA:	LPS-responsive beige-like anchor
NK:	Natural killer
PID:	Primary immunodeficiency disorder
PMA:	Phorbol 12-myristate 13-acetate
RAG:	Recombination-activating gene
SCID:	Severe combined immunodeficiency
STAT:	Signal transducer and activator of transcription
TCR:	T-cell receptor
TLR:	Toll-like receptor
Vβ:	Variable β
WAS:	Wiskott-Aldrich syndrome
WASP:	Wiskott-Aldrich syndrome protein
XLT:	X-linked thrombocytopenia

Flow cytometry is a routinely available laboratory method to study cells in suspension, including peripheral blood, bone marrow, cerebrospinal fluid, and other body fluids or tissue suspensions. The clinical application of flow cytometry evolved as a tool for enumeration of $CD4^+$ T cells in the blood of patients with HIV infection and to characterize hematologic malignancies. More recently, the role of flow cytometry has broadened to include the study of disorders of the immune system, including primary immunodeficiency disorders (PID). This review describes the use of flow cytometry in identifying cell-surface markers to characterize cells of the adaptive and innate immune system and then focuses on alternative applications of flow cytometry in clinical immunology, including evaluation of intracellular characteristics and immune cell function.

EVALUATION OF CELL-SURFACE PROTEINS

Evaluation for the presence or absence of multiple cell-surface markers by using polychromatic flow cytometry constitutes the basis of clinical immunophenotyping. This allows for the characterization of cell populations and subpopulations, identification of the status of cell differentiation, and quantification of surface proteins associated with specific cellular functions. Determination of immunophenotypic characteristics of T, B, and natural killer (NK) cell subsets by using flow cytometry allows classification of patients with severe combined immunodeficiency (SCID) into different categories that have historically narrowed the search for possible underlying genetic defects.[1,2] T-cell subpopulation characterization might also be useful in the setting of abnormal results on newborn screening for severe T-cell immunodeficiency.[1] When low or absent numbers of T-cell receptor (TCR) excision circles are detected at birth, immunophenotyping to evaluate for numbers of naive T cells based on CD45RA in combination with CD31, CD127, and/or CD62 ligand (CD62L) is typically a part of the follow-up evaluation. In addition, both hypomorphic SCID mutations and typical SCID associated with maternal engraftment result in circulating nonnaive T cells that have upregulated activation markers, such as HLA-DR and CD69.[2] Other surface antigens that are found only on T-activated cells are receptors for specific growth factors, such as CD25; receptors for critical elements required for cell growth, such as the transferrin receptor (CD71); and ligands for cell-to-cell communication after cell activation, including CD40 ligand (CD40L) on activated $CD4^+$ T cells.

Flow cytometry is important in the diagnosis of autoimmune lymphoproliferative syndrome (ALPS) because increased

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numbers of double-negative T (DNT) cells expressing the α/β TCR present in peripheral blood and lymphoid tissues is a diagnostic marker of the disease. These patients usually have mutations in genes that regulate the extrinsic Fas-mediated cell death pathway (*FAS*, *FASL*, and *CASP10*), and DNT cells homogeneously express multiple surface markers, including CD45RA, CD57, CD27, CD28, perforin, and HLA-DR, but lack CD45RO and CD56. This finding contrasts with DNT cells in peripheral blood of healthy control subjects, which predominantly express the γ/δ TCR receptor.[3]

Flow cytometry is a crucial diagnostic tool for MHC class II deficiency. This is a rare autosomal recessive form of PID characterized by the deficiency of different MHC class II molecules.[4] These defects affect the cellular and humoral immune responses by impairing the development of $CD4^+$ T_H cells and T_H cell–dependent antibody production by B cells.[5] Affected children typically present with severe respiratory and gastrointestinal tract infections, and hematopoietic stem cell transplantation is the only curative therapy available.[4] Complete absence of HLA-DR expression on B cells and monocytes by using flow cytometry is diagnostic of this disorder.[4,5]

Application of B-cell immunophenotyping has emerged as a useful tool in the evaluation of common variable immunodeficiency (CVID), a heterogenous group of disorders characterized by hypogammaglobulinemia, impaired antibody production, increased susceptibility to sinopulmonary infections, and immune dysregulation and cancer.[6] Patients with CVID often have normal or low numbers of B cells. However, evaluating B cells based on the distribution of naive ($CD27^-IgD^+IgM^+$), nonswitched memory ($CD27^+IgD^+IgM^+$), and switched memory ($CD27^+IgD^-IgM^-$) cells has proved to be a useful tool in categorizing patients with CVID.[7-10] Furthermore, immunophenotyping of these patients can be extended to various B-cell subsets beyond naive and memory cells to include marginal zone B cells ($CD19^+CD27^+IgM^+IgD^+$), transitional B cells ($CD19^+CD27^-CD24^{hi}CD38^{hi}IgM^{hi}CD10^+$), plasmablasts ($CD19^+CD20^-IgM^-CD38^{int}CD27^+$), and B cells with different levels of CD21 expression.[10]

A subgroup of CVID has been associated with abnormalities in the inducible T-cell costimulator, CD19, and B-cell activating factor receptor (TNFRSF13C) molecules. Screening for these defects is performed by using flow cytometry to detect reduced upregulation of inducible T-cell costimulator on activated T cells, or reduced expression of B-cell activating factor receptor or CD19 on B cells.[11-13]

Decreased numbers of switched memory B cells are also observed in patients with hyper-IgM (HIGM) syndromes.[14] These syndromes represent a group of genetic disorders affecting molecules involved in B-cell class-switch recombination and somatic hypermutation.[15,16] Affected patients present with normal or increased serum IgM levels and low levels of IgG and IgA. Although mutations in several genes have been associated with HIGM, the most frequently affected gene is the X-linked *CD40L* gene. Because CD40L (CD154) is important for normal T-cell function, deficient patients have not only bacterial but also opportunistic infections and malignancies.[17] CD40L expression by activated $CD4^+$ T cells is absent or reduced in approximately 80% to 90% of the patients when assessed with anti-CD40L–specific mAbs, whereas the remaining patients have mutations that result in expression of nonfunctional protein that is still detected by using the mAbs.[16] Flow cytometric detection can be further improved by using a biotinylated CD40-immunoglobulin fusion protein to detect functional CD40L, identifying more than 90% of the patients with confirmed *CD40L* mutations.[17] CD40 deficiency, one of several autosomal recessive HIGM syndromes, is a clinical phenocopy of CD40L deficiency that can be identified by assessing for CD40 expression on B cells, monocytes, and/or dendritic cells.[18] The remaining classic HIGM syndromes involve B-cell proteins that currently cannot be detected by using flow cytometry.

Genetic defects affecting Toll-like receptor (TLR) pathways have been described in immunodeficient patients with unique patterns of infection.[19] These include *IRAK4* mutations in patients with recurrent and/or severe pneumococcal infections, *NEMO* and *IKBA* defects in patients with atypical mycobacteriosis and other bacterial infections, and *UNC93B* and *TLR3* mutations in children with *Herpes simplex* encephalitis.[19] A reliable screening method for some of these diseases is a flow cytometric assay based on L-selectin (CD62L) shedding after TLR stimulation. In this method incubation of whole fresh blood with various TLR ligands induces rapid shedding of CD62L from the surfaces of granulocytes. The absence of shedding can identify patients with *IRAK4* and *UNC93B* mutations. Importantly, this procedure is not useful to identify patients with *TLR3*, *NEMO*, and *IKBA* mutations.[20]

Leukocyte adhesion deficiency (LAD) type 1 is associated with recurrent skin and deep-seated bacterial infections. It is caused by a defect in β_2-integrin expression that can be diagnosed by using flow cytometry, evaluating for the expression of cell-surface CD18 and its partner proteins, CD11a, CD11b, and CD11c.[21] In patients with LAD type 1, CD18 is usually less than 5% to 10% of normal values, and unlike control subjects, the level of expression is not upregulated after neutrophil activation. By contrast, in patients with LAD type 2 associated with defective fucosylation, the diagnosis can be suggested by demonstrating failure of CD15s (sialyl-Lewis X antigen) expression together with red blood cell typing that demonstrates the rare Bombay blood type.[21,22]

Cell-surface staining can also allow for investigation of lymphocyte clonality. T-cell clonality studies are particularly useful in evaluation of PIDs associated with a restricted T-cell repertoire. Omenn syndrome, a form of leaky SCID, is associated with multiple different genetic defects that impair but do not completely abrogate T-cell generation.[23,24] This disorder is characterized by oligoclonal T-cell expansion that can be detected in peripheral blood, as well as in T cells infiltrating the skin, liver, spleen, and lymph nodes.[24] Similarly, a restricted T-cell repertoire is seen in patients with SCID with maternal engraftment.[25] The method uses mAbs directed at specific T-cell antigen receptor variable β (Vβ) chains (Fig 1). This approach identifies significant underrepresentation or overrepresentation of a specific Vβ chain family expressing T cells and is complementary to PCR–based TCR spectratyping.[26]

In the setting of hematologic malignancies, issues of monoclonality can be fully or partially addressed by using flow cytometry when analyzing B-cell malignancies and, in some circumstances, when studying T-cell disease. Normally, B cells are a heterogeneous mixture of mutually exclusive κ or λ light chain–expressing cells. Measuring the distribution of κ or λ light chain–expressing B cells or plasmacytes can be informative with respect to the presence or absence of monoclonality.[27] The capacity to evaluate T-cell monoclonality by using flow cytometry is less definitive and consists of using the method described

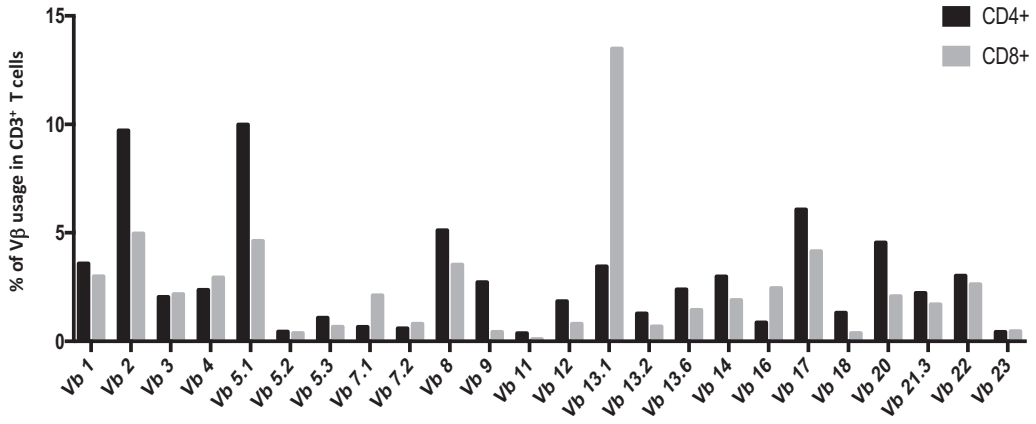

FIG 1. Flow cytometric analysis of 24 TCR Vβ families in $CD4^+$ *(black columns)* and $CD8^+$ *(gray columns)* cells in a healthy control subject. The different families are represented showing a normal polyclonal T-cell repertoire distribution. *X-axis,* Single Vβ families; *y-axis,* frequency of Vβ use in $CD3^+$ TCR α/β T cells.

previously to identify evidence of significant overrepresentation of one Vβ family. This is an indirect measure that suggests possible clonality. Traditional flow cytometry testing for Vβ chain expression requires 8 tubes (24 Vβ-specific mAbs), but a more recently described method combines all TCR Vβ mAbs into 1 tube.[28] This latter technique enables linking the aberrant immunophenotype of a neoplastic T-cell clone to its apparent clonal TCR Vβ restriction. A false-positive rate for identifying a clonal population with this approach was estimated to be less than 6.2%.[28] Despite these results, molecular approaches for T-cell clonality by using RNA sequencing have increased sensitivity and diagnostic utility compared with flow cytometry.[29]

Additionally, cell surface–based flow cytometry is able to detect rare events, including detecting $CD34^+$ hematopoietic stem cells in peripheral blood under resting conditions[30] and evaluating for minimal residual disease in patients with hematologic malignancy, such as hairy cell leukemia.[31]

Despite advances in the evaluation of cell-surface markers (Table I), this approach has limitations because it does not assess the functional status of cells. For example, in patients with CVID, the presence of normal B-cell numbers does not correlate with immunoglobulin production and an antigen-specific antibody response.[32] Likewise, in patients with SCID caused by X-linked common γ chain defects, normal surface protein expression of this protein does not rule out a disease-causing mutation, resulting in expression of a defective protein incapable of initiating a signal, a consistent problem with immunoassay-based protein detection, such as flow cytometry.

TABLE I. Evaluation of PIDs by using flow cytometry

Cell-surface protein staining
Low or absent TRECs on NBS: naive T cells and recent thymic emigrants
MHC class II deficiency: absent HLA-DR
ALPS: increased α/β TCR double-negative ($CD4^-CD8^-$) T-cell counts
Omenn syndrome and SCID: restricted T-cell repertoire
CVID: CD19 (B cells), BAFF-R (B cells), ICOS (activated T cells)
CVID: decreased switched memory B-cell counts
X-linked HIGM: CD40L (activated T cells)
Autosomal recessive HIGM: CD40 (B cells)
LAD type 1: CD18 (granulocytes)
LAD type 2: CD15s
IRAK4 and UNC93B deficiency: CD62L (granulocytes)
Intracellular protein staining
XLA: BTK (monocytes, platelets)
WAS, WIP deficiency: WASp (lymphocytes, myeloid cells)
XLP1/2: SAP (CD8 T cells, NK cells)/XIAP (lymphocytes)
FHL: perforin (CD8 T cells, NK cells),
IPEX: FoxP3 (regulatory T cells)
CTLA4 haploinsufficiency: CTLA4 expression (regulatory T cells)
LRBA deficiency: CTLA4 expression (regulatory T cells), LRBA expression (PBMCs)
DOCK8 deficiency: DOCK8 expression

ALPS, Autoimmune lymphoproliferative syndrome; *BTK*, Bruton tyrosine kinase; *FHL*, familial hemophagocytic lymphohistiocytosis; *FoxP3*, forkhead box P3; *ICOS*, inducible T-cell costimulator; *IPEX*, immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome; *IRAK4*, IL-1 receptor–associated kinase 4; *LRBA*, LPS-responsive beige-like anchor; *NBS*, Newborn screening; *SAP*, Signaling lymphocyte activation molecule-associated protein; *TREC*, T-cell excision circle; *WIP*, WASP-interacting protein; *XIAP*, X-linked inhibitor of apoptosis; *XLA*, X-linked agammaglobulinemia; *XLP*, X-linked lymphoproliferative syndrome.

EVALUATION OF INTRACELLULAR PROTEINS

Clinical flow cytometry now includes the capacity to identify and quantify intracellular proteins associated with immune function (Table I). This method requires fixation and permeabilization to allow the mAb to pass through the cell membrane. A clinical application of this approach is demonstrated by screening of patients for Wiskott-Aldrich syndrome (WAS).[33] Either the absence or decreased intracellular expression of the Wiskott-Aldrich syndrome protein (WASP) can confirm the diagnosis of WAS and/or X-linked thrombocytopenia (XLT; Fig 2).[34] Additionally, this testing can also identify carriers of WAS and XLT.[35] However, the presence of normal intracellular WASP expression, as determined by using flow cytometry, does not rule out WAS/XLT because some patients express a dysfunctional protein detectable at levels comparable with those of control subjects.[33]

Evaluation of WASP is also an effective test for WASP-interacting protein deficiency syndrome, an autosomal recessive disease with clinical features similar to WAS caused by impaired expression of WASP-interacting protein, resulting in WASP degradation.[36] This method has also proved useful in monitoring the presence of somatic reversion of the *WAS* gene[37] and evaluation of donor chimerism after hematopoietic stem cell

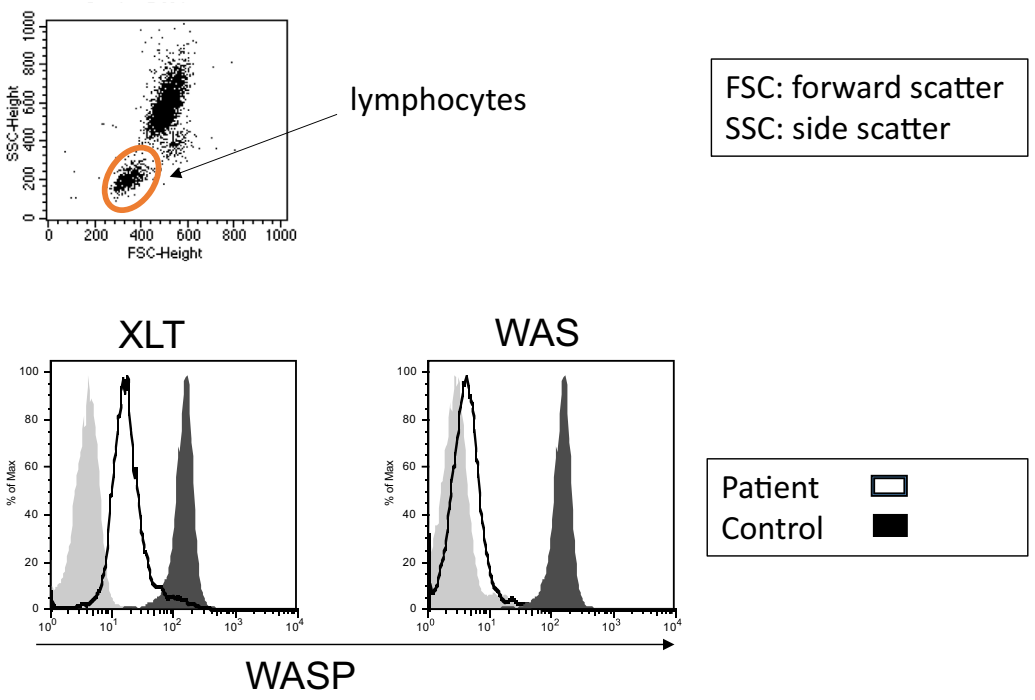

FIG 2. Flow cytometric detection of WASP in a patient with WAS and a patient with XLT. Cytoplasmic WASP expression was markedly reduced in patients' lymphocytes. *Gray-shaded histogram*, Isotype control; *black line*, patients with XLT and WAS; *black-shaded histogram*, control subjects.

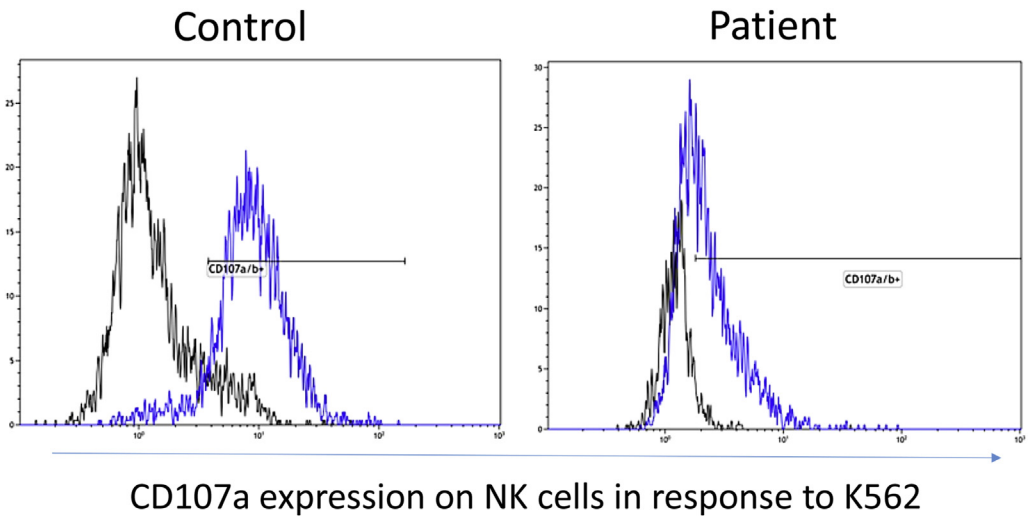

FIG 3. Flow cytometric detection of CD107a expression on NK cells as a marker of degranulation. PBMCs were cultured in the absence or presence of target cells (K562). After 4 hours of culture, cells were stained with mAbs to CD107a, CD3, and CD56 to allow for evaluation of CD107a expression on $CD3^{-}CD56^{+}$ NK cells demonstrating CD107a expression after NK cell activation. *Black line*, Unstimulated NK cells; *blue line*, NK cells cocultured with K562 cells.

transplantation.[38] Post-transplantation WASP-positive cells of lymphoid or myeloid lineage in protein-negative patients with WAS represent donor cells, allowing for accurate assessment of the degree of chimerism among these various cell types.[38]

A further example of intracellular protein testing by using flow cytometry in patients with PIDs involves screening for patients with possible immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. This diagnosis is confirmed in male patients whose $CD4^{+}CD25^{+}$ regulatory T cells demonstrate an absence of forkhead box P3 protein expression.[39]

Cytotoxic T lymphocyte–associated protein 4 (CTLA4 [CD152]) is another intracellular marker that can be routinely assessed by using flow cytometry in selected patients with CVID-like features, as well as severe enteropathy, brain lesions, and autoimmune cytopenias.[40,41] CTLA4 is an inhibitory molecule expressed by activated T cells that binds to CD80/CD86 on the

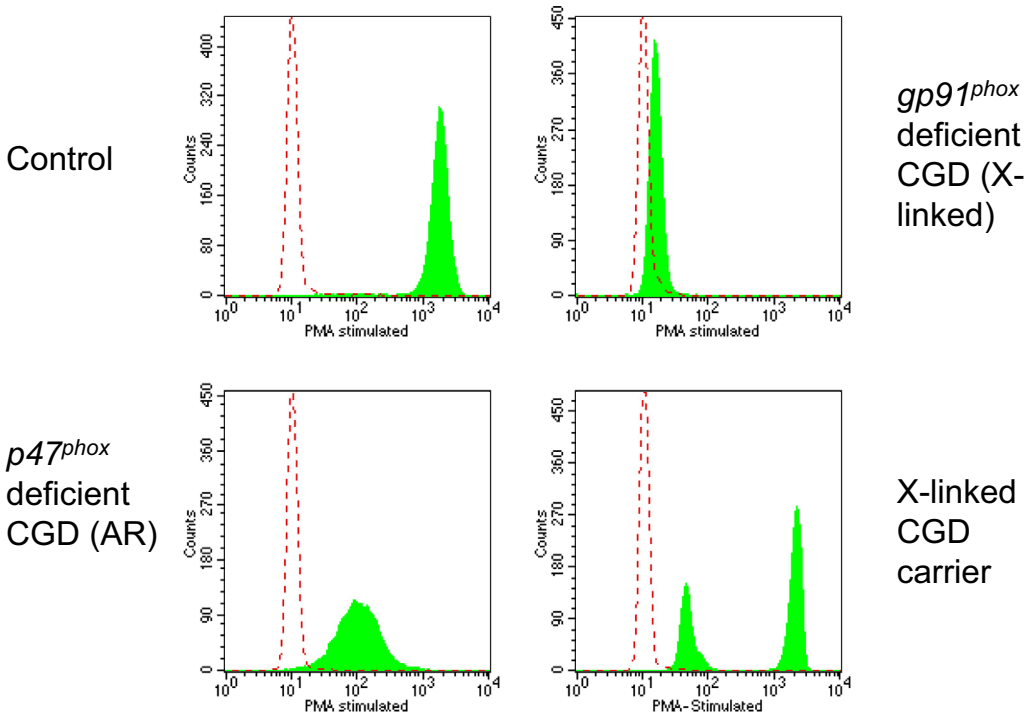

FIG 4. Flow cytometric analysis of DHR oxidation in control subjects, gp91phox-deficient (X-linked) patients, p47phox-deficient (autosomal recessive *[AR]*) patients with CGD, and an X-linked maternal carrier. In the assay DHR-loaded granulocytes were analyzed before *(red dashed line histogram)* and after *(green histogram)* stimulation with PMA.

surfaces of antigen-presenting cells and represents one means of downregulating an immune response. Patients with CTLA4 haploinsufficiency have reduced intracellular expression of CTLA4 in regulatory T cells. Similarly, patients with biallelic loss-of-function mutations in the LPS-responsive beige-like anchor gene *(LRBA)* have reduced expression of CTLA4.[42,43] This is linked to the finding that LRBA colocalizes with CTLA4 in endosomal vesicles, and in the setting of LRBA deficiency, CTLA4 recycling is augmented, resulting in reduced levels of protein. The phenotypic similarity between LRBA and CTLA4 deficiencies might be explained by this common defect in CTLA4 expression.[44] Surface expression of CTLA4 protein can also be detected on T cells after activation with IL-2.[45] More recently, a flow cytometric test aimed to detect intracellular LRBA protein expression on PBMC stimulation has been implemented. This method showed 94% sensitivity and 80% specificity in identifying LRBA-deficient patients.[46]

Additional examples of intracellular protein detection as a screening test for PIDs include intracellular staining for Bruton tyrosine kinase protein for possible X-linked agammaglobulinemia. Bruton tyrosine kinase expression is evaluated in monocytes or platelets because these patients have absent or markedly decreased numbers of circulating B cells.[47] 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.[48,49] 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.[50] The lack of intracellular perforin expression in NK cells (and $CD8^+$ T cells) is characteristic of familial hemophagocytic lymphohistiocytosis type 2.[51]

Additionally, intracellular flow cytometry allows for screening of patients with severe atopic dermatitis, viral and bacterial infections, increased IgE levels, reduced T- and B-cell counts, and impaired antibody responses for deficiency in the dedicator of cytokinesis 8 (DOCK8) protein. DOCK8 is expressed intracellularly in lymphoid and myeloid cells[52] and can be analyzed by using a 2-step flow cytometric method.[52,53] Levels of expression of the protein can vary, and thus it is important to compare the mean fluorescence intensity of DOCK8 staining between healthy control subjects and patients.

EVALUATION OF CELLULAR FUNCTION

For both surface and intracellular staining, it is important to recognize that the absence of protein expression is diagnostic of a specific defect, as previously noted; however, detection of protein can be misleading when associated with expression of a mutant protein that is functionally abnormal. This issue usually can be resolved further by functional testing, and flow cytometry provides a number of different immune function techniques, including assessment of immune cell lineage-specific functions, cell activation, cell proliferation, and T_H cell cytokine production.

Lineage-specific function testing includes evaluation of NK cell degranulation by assessing CD107a surface expression after stimulation with an appropriate target cell line (eg, K562) or phorbol 12-myristate 13-acetate (PMA) plus ionomycin. CD107a is normally expressed on cytoplasmic granules, but after incubation with K562 target cells or exposure to PMA/ionomycin, the

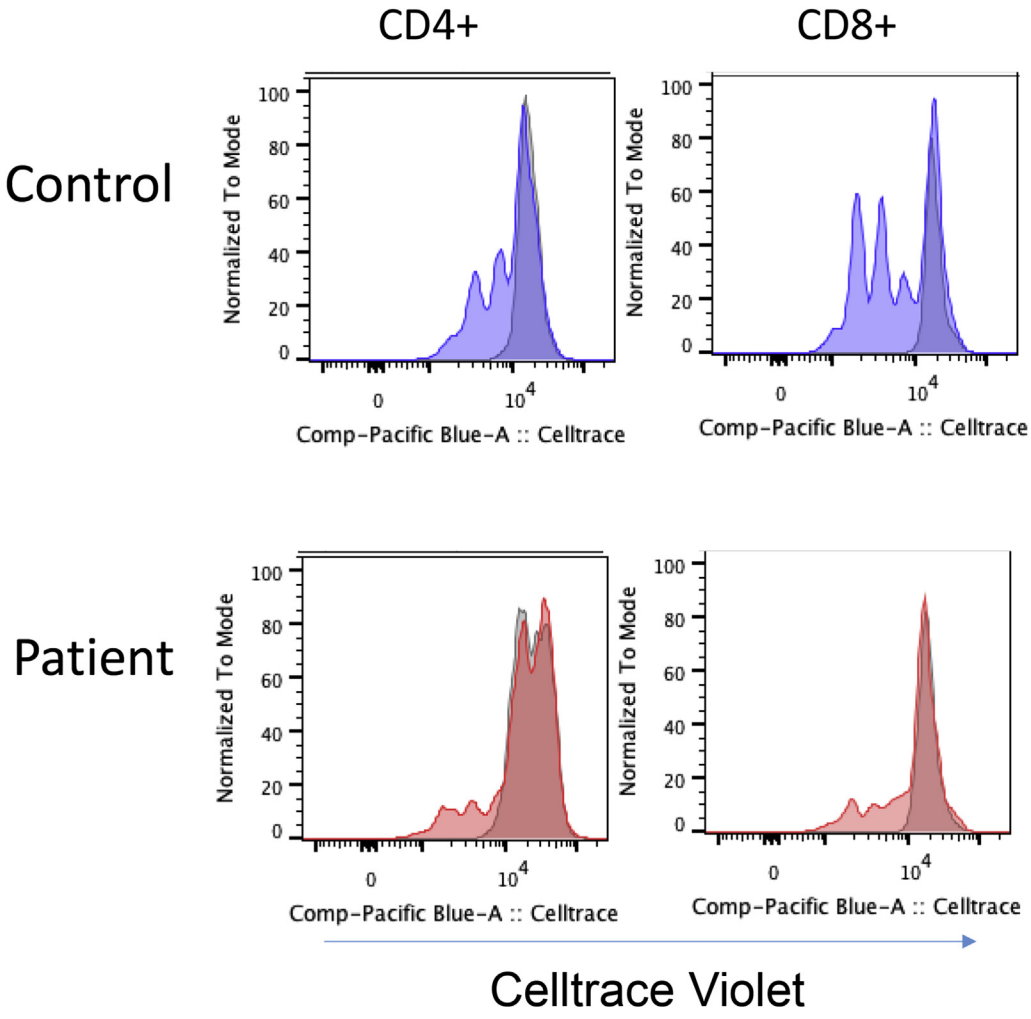

FIG 5. Flow cytometric analysis of cell division using Cell Trace Violet–loaded mononuclear cells from a control subject *(purple histogram)* and an immunodeficient patient *(red histogram)*. In this assay $CD4^+$ and $CD8^+$ T lymphocytes were evaluated after cell stimulation with anti-CD3 and anti-CD28 mAbs. Each peak of decreased fluorescence represents a round of cell division as a result of a 50% decrease in the amount of cell tracking dye in each daughter cell.

protein becomes detectable on the surface of more than 10% of NK cells (Fig 3).[54] This assay serves as an adjunct to the traditional NK cell cytotoxicity assay and is particularly useful in screening for the diagnosis of familial hemophagocytic lymphohistiocytosis. Specifically, a lack of CD107a surface expression after incubation with K562 target cells is consistent with syntaxin-11, mammalian uncoordinated 13.4, syntaxin-binding protein 2, or Rab27A protein defects.[54-57]

Another important cell lineage-specific functional flow cytometric assay uses the hydrogen peroxide–sensitive dye dihydrorhodamine 123 (DHR) to evaluate oxidative burst in neutrophils and other phagocytic cells. This has proved to be a sensitive diagnostic test for chronic granulomatous disease (CGD). The method involves loading neutrophils with DHR, stimulating the cells with PMA for a short period, and evaluating after activation for increased cellular fluorescence.[58] It is a sensitive assay to identify even minimal oxidase activity and has proved to be a reliable method to follow donor chimerism after allogeneic stem cell transplantation and to identify corrected cells after gene therapy. In addition, it is an effective method to assess X-linked female CGD carriers demonstrating the distribution of normal versus mutant neutrophils caused by lyonization (Fig 4).[59] Recently, the severity of the oxidative defect assessed by using the DHR flow assay has been correlated with patient outcome, and the distribution of normal versus mutant neutrophils in X-linked carriers has been shown to be a strong predictor of infectious risk.[58-60]

Although the DHR assay is considered the standard method for the diagnosis of CGD, this test has some limitations. Low test sensitivity has been associated with *in vitro* artifacts, inappropriate sample handling, and neutropenia. Furthermore, CGD-unaffected subjects with ongoing severe infections or inflammation can have exhausted neutrophils with impaired metabolic function, thus leading to falsely decreased DHR assay results.[61]

Evaluation of cellular activation using flow cytometry can include assessment of surface proteins (eg, CD69, CD25, and CD71), as well as intranuclear proteins (eg, Ki-67 and proliferating cell nuclear antigen), which are upregulated after activation. Cell proliferation induced by an activation signal is a crucial parameter in the diagnosis of patients with a possible T-cell defect, including infants with an abnormal newborn screening test

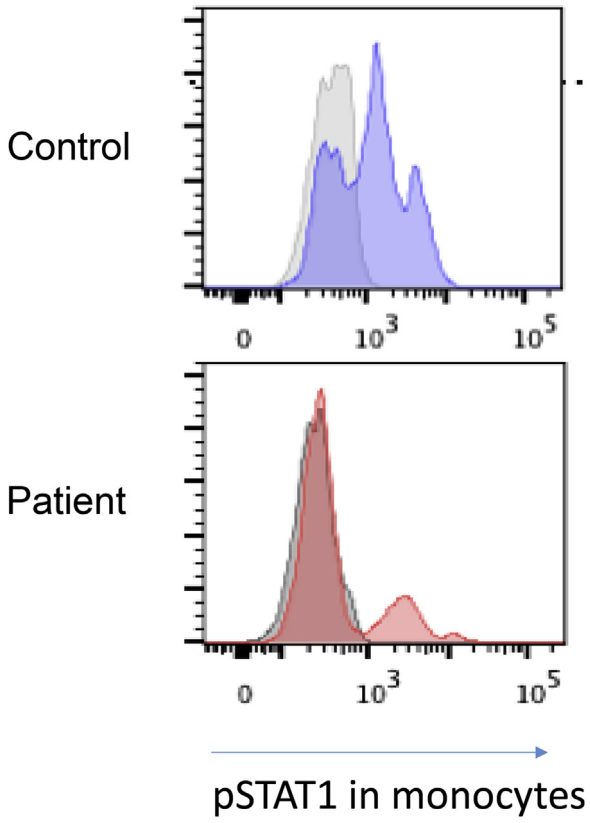

FIG 6. Flow cytometric analysis of STAT1 phosphorylation in monocytes after 20 minutes of stimulation with IFN-γ in control cells *(purple histogram)* and patients' cells (defect in the IFN-γ receptor; *red histogram*). *Solid gray histogram,* Isotype control.

result for SCID. T-cell proliferation can be measured by using flow cytometry with a lipophilic membrane dye (eg, carboxyfluorescein succinimidyl ester and Cell Trace Violet), also referred to as a cell tracking dye. In control subjects this assay demonstrates successive loss of fluorescence (50% decrease) for each round of cell division (Fig 5).[62] An alternative approach to evaluate lymphocyte proliferation after cell activation relies on the nucleotide analogue EdU. In this method detection of DNA synthesis depends on copper-catalyzed click chemistry, which leads to EdU covalently binding a fluorescent azide. The results of this assay are expressed as the percentage of cells (eg, $CD3^+$ T cells) that show increased fluorescence compared with unstimulated cells.[63] Both approaches enable assessing *in vitro* cell proliferation at the cell population or subpopulation level after mitogen (72 hours) or recall antigen (6-7 days) stimulation when used in combination with cell-surface protein assessment (eg, CD3, CD4, and CD8).

Flow cytometry can be used to detect phosphorylated intracellular proteins associated with specific activation signals as an alternate functional test for cell activation. An example is the detection of phosphorylated signal transducer and activator of transcription (STAT) 5 after IL-2 or IL-7 stimulation of T cells. This is particularly useful in the context of clinical settings suggestive of T-cell dysfunction. Similarly, flow cytometry allows for the detection of STAT1 phosphorylation after IFN-γ stimulation of monocytes, an approach that can help to screen for PIDs caused by impairment of IFN-γ receptor function, as well as loss-of-function or gain-of-function mutations in STAT1 (Fig 6).[64]

Evaluation of the mammalian target of rapamycin intracellular signaling pathway based on phosphorylation of the proteins AKT and S6 is a useful screening approach for diagnosing activated phosphoinositide 3-kinase δ syndrome type 1 and 2. Increased levels of phosphorylated AKT and S6 are associated with a gain of function in the mammalian target of rapamycin pathway caused by mutations in *PIK3CD* and *PIK3R1*, resulting in activated phosphoinositide 3-kinase δ syndrome type 1 or 2, respectively.[65,66] Another example is infantile-onset multisystem autoimmune disease 1 caused by heterozygous gain-of-function mutations in *STAT3*.[67] Those patients can have increased STAT3 phosphorylation in unstimulated lymphocytes.[68]

Overall, phosphorylation assays have proved useful in the assessment of gain-of-function mutations in which there can be increased phosphorylation or delayed dephosphorylation of specific mutant proteins or downstream molecules linked to the mutant protein that is inducing augmented activity of a specific pathway. A number of additional intracellular signaling proteins that undergo phosphorylation after a specific activation signal can be assessed with flow cytometry by using commercially available reagents.

Flow cytometry can be applied for functional characterization of a mutant protein based on cell transfection of mutant constructs that carry green fluorescent protein (GFP) or other fluorescent protein sequences. An example is assessment of recombination-activating gene (RAG) protein recombination activity. Lack of RAG protein expression in circulating mature T and B cells represents a major hurdle to study the functional effects of *RAG* missense mutations. Development of *in vitro* assays based on introduction of RAG mutant constructs into Abelson virus–transformed $Rag1^{-/-}$ (or $Rag2^{-/-}$) pro-B cells containing an inverted GFP cassette flanked by recombination signal sequences, allows for flow cytometry–based analysis of GFP expression as a readout of RAG recombination activity.[69,70]

Another functional application of flow cytometry is detection of intracellular cytokines after *in vitro* cell activation with PMA and ionomycin or a specific recall antigen to characterize specific T_H subsets (eg, T_H1, T_H2, and T_H17).[71] Within PIDs, this has proved useful in identifying alterations in generation of specific T_H subsets. For example, in patients with Job syndrome, a defect in T_H17 generation was identified as at least 1 explanation for the presence of chronic mucocutaneous candidiasis (Fig 7).[72] This technology has also been applied in the setting of allergic disease to characterize the specific T_H cell response to allergens, including evaluation of lavage cells and cell suspensions from tissue samples after *in vivo* challenge.

Flow cytometry has some definitive limitations, including the need for fresh specimens, an observation that requires care to evaluate appropriate controls in the setting of shipped samples. Medications or comorbidities can be significant confounders in the interpretation of results. Handling of the samples and assay execution are operator dependent. In addition, optimizing the control subject (and/or shipping control) to match the patient's age and sex is not always possible. Regardless of the control sample used, pediatric results should always be compared with available age-matched reference intervals. Furthermore, comparison of results over time and across platforms can be challenging; however, the unit of fluorescence intensity, molecules of equivalent soluble fluorochrome, is considered a useful tool to standardize flow cytometric analysis of cellular protein expression.[73]

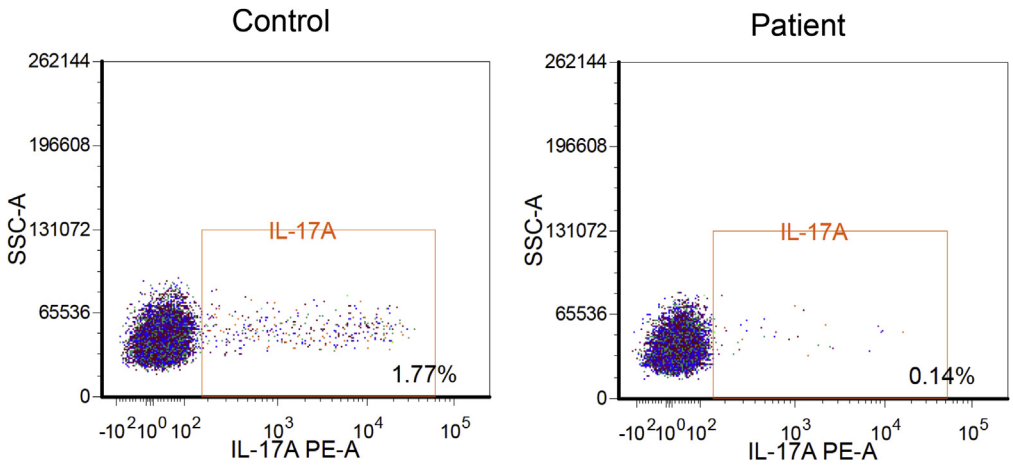

FIG 7. Flow cytometric quantification of intracellular IL-17A production in $CD4^+$ T cells of control subjects *(left)* and patients *(right)* after induction with PMA and ionomycin. The patient shows a decreased percentage of IL-17A$^+$ cells, indicating reduced generation of T_H17 cells.

The limitation of analyzing multiple cellular markers with flow cytometry because of the lack of an extensive repertoire of clinically available fluorochromes together with compensation challenges must also be considered. These issues have been overcome by a new technology called mass cytometry (CyTOF),[74] which uses rare earth lanthanide-labeled antibodies as detecting reagents and allows for analysis of more than 40 parameters on a cell-by-cell basis.[75-77] However, the cellular throughput is lower (500-1,000 cells/s) compared with that when using a conventional flow cytometer (10-50,000 cells/s).

An additional potential limitation of this technology in patients with PIDs is the relatively large cell acquisition requirement, which can be difficult to achieve, particularly in samples from young pediatric subjects. Although mass cytometry has not yet gained access as part of routine diagnostic flow cytometry in the clinical immunology laboratory, it is possible that future improvements/developments in this technology will make it more suitable for clinical purposes.[78,79]

CONCLUSION

The application of flow cytometry as a clinical laboratory method has evolved from the identification of cell-surface proteins to characterizing intracellular proteins and providing multiple different approaches to assess immune function. This expanded menu of flow cytometry has increased the utility of this platform in characterizing and diagnosing immunologic disorders.

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