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Using Flow Cytometry to Screen Patients for X-linked Lymphoproliferative Disease Due to SAP Deficiency and XIAP Deficiency

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Abstract

X-linked lymphoproliferative disease is a rare congenital immunodeficiency that is most often caused by mutations in *SH2D1A*, the gene encoding signaling lymphocyte activation molecule (SLAM)-associated protein (SAP). XLP caused by SAP deficiency is most often characterized by fulminant mononucleosis/EBV- associated hemophagocytic lymphohistiocytosis (HLH), lymphoma, and dysgammaglobulinemia. XLP has also been found to be caused by mutations in *BIRC4*, the gene encoding X-linked inhibitor of apoptosis (XIAP). Patients with XIAP deficiency often present with HLH or recurrent HLH, which may or may not be associated with EBV. XLP is prematurely lethal in the majority of cases.

While genetic sequencing can provide a genetic diagnosis of XLP, a more rapid means of diagnosis of XLP is desirable. Rapid diagnosis is especially important in the setting of HLH, as this may hasten the initiation of life-saving medical treatments and expedite preparations for allogeneic hematopoietic cell transplantation (HCT).

Flow cytometry offers a means to quickly screen patients for XLP. Flow cytometry can be used to measure lymphocyte SAP or XIAP protein expression, and can also be used to observe lymphocyte phenotypes and functional defects that are unique to XLP. This review will give a brief overview of the clinical manifestations and molecular basis of SAP deficiency and XIAP deficiency, and will focus on the use of flow cytometry for diagnosis of XLP.

Keywords

X-linked lymphoproliferative disease; hemophagocytic lymphohistiocytosis; XIAP; SAP; BIRC4; SH2D1A

Introduction

X-linked lymphoproliferative disease (XLP; Mendelian Inheritance in Man [MIM] 308240) is a rare congenital immunodeficiency that was first described in 1975.(Purtilo et al., 1975) It is a disease characterized most often by fulminant mononucleosis/EBV- associated

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hemophagocytic lymphohistiocytosis, lymphoma, and dysgammaglobulinemia.(Seemayer et al., 1995) XLP is prematurely lethal in the majority of cases, often due to EBV-associated HLH.(Seemayer et al., 1995) The prevalence of XLP has been estimated at 2–3 per million males.(Purtilo and Grierson, 1991) though the frequency may be under-reported due to a variety of reasons including failure to recognize the disorder.

While clinical criteria exist for the diagnosis of patients with XLP, a genetic diagnosis was not possible until 1998 when it was discovered that the majority of cases of XLP are caused by mutations in *SH2D1A*, the gene encoding signaling lymphocyte activation molecule (SLAM)-associated protein (SAP).(Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998) More recently, in 2006, mutations in *BIRC4* (encoding X-linked inhibitor of apoptosis, XIAP) were discovered in a minority of patients with XLP phenotypes (Rigaud et al., 2006). Because of these discoveries, a definitive genetic diagnosis is now possible in many patients with XLP phenotypes. Unfortunately, genetic studies often require several weeks to be completed. A rapid means of diagnosis of XLP and related disorders is desirable, especially in the setting of severe mononucleosis/hemophagocytic lymphohistiocytosis (HLH), as a clear diagnosis may hasten the initiation of life-saving medical treatments, as well as expedite preparations for allogeneic hematopoietic cell transplantation (HCT). The ability to use flow cytometry to quickly measure lymphocyte SAP or XIAP protein expression, or to observe lymphocyte phenotypes and functional defects that are unique to XLP, can facilitate a rapid diagnosis. These studies can also aid in the interpretation of genetic results when new or unreported sequence variants are encountered.

This review will give a brief overview of the clinical manifestations and molecular basis of SAP deficiency and XIAP deficiency, and will highlight the immunologic abnormalities that are unique to these disorders which can be exploited for use in patient screening with flow cytometry.

Clinical manifestations and molecular basis of SAP deficiency and XIAP deficiency

SAP deficiency

XLP is most often caused by deficiency of SLAM-associated protein (SAP) due to mutations in the *SH2D1A* gene found on chromosome Xq24–25.(Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998) SAP is a 128-amino acid protein involved in the intracellular signaling of the SLAM (signaling lymphocyte activation molecule) family of receptors.(Ma et al., 2007) XLP due to SAP deficiency usually presents in childhood or adolescence, and clinical manifestations include fulminant infectious mononucleosis/EBV-associated HLH (in ~60% of cases), lymphoproliferative disease including malignant lymphoma (~30%), hypo-/dys-gammaglobulinemia (~30%), and aplastic anemia (3%).(Seemayer et al., 1995) Lymphomas are typically of B-cell origin (non-Hodgkin's) and often occur in extra-nodal sites, particularly the ileocecal region.(Harrington et al., 1987) Some patients with hypo-/dysgammaglobulinemia may be initially diagnosed as having common variable immune deficiency.(Soresina et al., 2002; Aghamohammadi et al., 2003) Lymphocytic vasculitis, macrophage activation syndrome (an HLH variant), interstitial pneumonitis, and encephalitis have also been observed.(Dutz et al., 2001; Kanegane et al., 2005; Snow et al., 2009; Talaat et al., 2009)

Loss of functional SAP causes several defects in lymphocyte function. In brief, SAP is necessary for normal T-cell-dependent humoral immune responses, NK cell and CD8+ T cell cytotoxicity, and development of invariant natural killer T (iNKT) cells (Ma et al., 2007). More recently, SAP was found to be necessary for sustained T cell:B cell interactions

that ensure proper germinal center formation and B cell help.(Qi et al., 2008; Cannons et al., 2010). Moreover, SAP is also required for T cell restimulation-induced cell death (RICD), a self-regulatory mechanism of apoptosis critical for T cell homeostasis.(Nagy et al., 2009; Snow et al., 2009). Although Epstein-Barr virus (EBV) has been historically identified as a triggering event for infectious mononucleosis and associated hemophagocytic lymphohistiocytosis (HLH); not all disease manifestations are associated with EBV, consistent with the presence of intrinsic lymphocyte defects.

XIAP deficiency

Deficiency of X-linked inhibitor of apoptosis (XIAP), caused by *BIRC4* gene mutations, was discovered to be associated with XLP phenotypes in 2006.(Rigaud et al., 2006) In contrast to SAP deficiency, over 90% of patients with XIAP deficiency develop hemophagocytic lymphohistiocytosis, with or without association with EBV, and recurrent HLH is common. (Rigaud et al., 2006; Marsh et al., 2010; Zhao et al., 2010) A minority of patients may display hypogammaglobulinemia, and no cases of lymphoma have been observed in patients with XIAP deficiency to date.

XIAP is an inhibitor of apoptosis (IAP) family member, consisting of 3 baculovirus IAP repeat (BIR) domains and a C-terminal RING (really interesting new gene) domain. XIAP is best known for its caspase-inhibitory and anti-apoptotic properties, and BIR2 and its N-terminal linker region inhibit caspase-3 and caspase-7, while BIR3 inhibits caspase-9.(Chai et al., 2001; Huang et al., 2001; Shiozaki et al., 2003; Scott et al., 2005) The BIR regions of XIAP can also interact with non-caspase proteins such as RIP2 and TAB1. These and other XIAP interactions mediate signaling pathways involving Nuclear Factor -kappa B (NF-κB), c-jun N-terminal kinase (JNK), NOD1 and NOD2, and the bone morphogenetic protein (BMP) receptors.(Sanna et al., 1998; Yamaguchi et al., 1999; Lewis et al., 2004; Kaur et al., 2005; Lu et al., 2007; Krieg et al., 2009) The RING domain possesses E3-ubiquitination function.(Yang et al., 2000; Galban and Duckett, 2010) Exactly why deficiency of XIAP leads to the development of HLH is not currently understood.

Using flow cytometry to screen patients for SAP and XIAP Deficiencies

Measurement of lymphocyte SAP and XIAP expression

SAP and XIAP expression can be measured by flow cytometry using standard methods which are previously reported (Figures 1 and 2). After fixation, whole blood lymphocytes are permeabilized and stained with monoclonal anti-SAP (clone KST-3)(Shinozaki et al., 2002) or anti-XIAP (clone 48, BD Biosciences)(Marsh et al., 2009b) antibodies that have been validated for use in flow cytometry, followed by fluorochrome-conjugated secondary antibody staining.(Shinozaki et al., 2002; Tabata et al., 2005; Marsh et al., 2009b) Appropriate surface marker staining allows characterization of individual lymphocyte subset protein expression. In our laboratory, T cells are defined as CD3+ lymphocytes and further categorized based on CD4 or CD8 expression. B cells are defined as CD3- CD19+ lymphocytes, and NK cells are defined as CD3- CD56+ lymphocytes. These standard surface markers are commercially available conjugated to a variety of fluorochromes, enabling laboratories to choose color combinations that suit the particular experience and capabilities of the laboratory and that also allow optimal detection of the intracellular proteins.

SAP can be readily detected in T cells and NK cells, and T cell expression is increased with activation.(Shinozaki et al., 2002; Tabata et al., 2005) As described in the literature, patients with SAP deficiency typically demonstrate markedly decreased or absent SAP expression. (Shinozaki et al., 2002; Tabata et al., 2005) One patient has uniquely been observed to

possess bimodal expression of SAP in CD8⁺ T cells, possibly due to the fact that this patient possesses an *SH2D1A* mutation at the splice acceptor site of exon 2, or, alternatively, possibly due to revertant mosaicism.(Tabata et al., 2005) Flow cytometric analysis of SAP can also be used for the detection of carrier status in many cases (Figure 1).(Tabata et al., 2005) If a suspected maternal carrier lacks a bimodal pattern of SAP expression, it is possible that the affected patient's mutation arose de novo. However, *SH2D1A* sequencing should additionally be performed for confirmation, as not all carriers can be definitively diagnosed with flow cytometry.

Flow cytometric detection of XIAP can also be used as a screening test for XIAP deficiency. XIAP has been found to be expressed in many human tissues, including all hematopoietic cells.(Duckett et al., 1996; Rigaud et al., 2006; Marsh et al., 2009b). XIAP is readily detectable in normal granulocytes, monocytes, and all lymphocyte subsets (Figure 2). Depending on the specific mutation, XIAP has been observed to be either absent or decreased in patients with XIAP deficiency.(Marsh et al., 2009b; Zhao et al., 2010) Mothers who are carriers of *BIRC4* mutations display bimodal distribution of XIAP, and interestingly are typically skewed towards XIAP-expressing cells in all subsets, indicating a likely survival advantage for XIAP-expressing cells.(Marsh et al., 2009b) Significant skewing can make the diagnosis of carrier status difficult in some cases, and *BIRC4* sequencing should be performed when a clearly bi-modal pattern is not evident. De novo mutations in *BIRC4* are also observed.

Flow cytometric measurement of XIAP can also be used for monitoring of lineage-specific donor chimerism in the setting of allogeneic hematopoietic cell transplantation (HCT) (Figure 3).(Marsh et al., 2009b) Similarly, flow cytometry can be used to monitor donor chimerism within the SAP-expressing lymphocyte populations in patients with SAP deficiency who have undergone allogeneic HCT.

Measurement of iNKT cell populations

Human invariant natural killer T cells (iNKT cells) are a population of T cells that express an invariantly rearranged T cell receptor (TCR) consisting of TCRV α 24 and TCRV β 11 chains which recognize glycosphingolipid antigens presented by the CD1d molecule. Following activation, iNKT cells can influence immune responses through secretion of a variety of cytokines which can either stimulate or suppress immunity. iNKT cells have been implicated in altering immune responses involved in protection from infection, auto-immunity, and tumors.(Godfrey and Kronenberg, 2004) This unique population is known to be absent in humans and also mice with SAP deficiency, due to a requirement of SAP for iNKT cell development.(Nichols et al., 2005; Pasquier et al., 2005) Thus, the use of flow cytometry to detect an absence of iNKT cells can be used as a screening test for SAP deficiency. However, iNKT cells can constitute as little as 0.008% of peripheral blood T cell populations in normal individuals,(Marsh et al., 2009a) and it must be understood that evaluation of peripheral blood iNKT populations constitutes "rare event" flow cytometry. (Donnenberg and Donnenberg, 2007; Roederer, 2008) Other than this, the methods are straightforward. iNKT cells are co-stained with fluorochrome-conjugated monoclonal antibodies against CD3, TCRV α 24, and TCRV β 11 following standard surface staining protocols (Figure 4).(Marsh et al., 2009a) Given that iNKT cells constitute such a small percentage of peripheral blood T cells, care must be taken to ensure that one is able to discern the rare positive events by flow cytometry. We have found that by acquiring 100,000 CD3⁺ lymphocyte events, we can comfortably discriminate the presence or absence of iNKT cells in peripheral blood specimens with comparison to CD3⁺ lymphocytes co-stained with 2 isotype controls in place of the TCRV α 24 and TCRV β 11 antibodies.(Marsh et al., 2009a)

While the original cohort of patients with XIAP deficiency were observed to possess decreased populations of iNKT cells,(Rigaud et al., 2006) later evaluation of patients in comparison to a large pediatric and adult control group found that iNKT cell populations in patients with XIAP deficiency are numerically within normal limits.(Marsh et al., 2009a) The discrepancy between reports may be related to the sizes of the comparison control groups, or may be related to the disease status of the patients at the time of evaluation. At the present time, the role of XIAP in the development of iNKT cells is not clearly defined. Thus, evaluation of iNKT cell populations is likely to only be of direct benefit for evaluation of patients for SAP deficiency.

Measurement of T-cell restimulation-induced cell death

T cell restimulation-induced cell death (RICD), which is also sometimes referred to as activation-induced cell death (AICD), refers to T cell receptor mediated apoptosis of mature, cycling T cells that occurs distal to initial T cell activation.(Snow et al., 2010) This phenomenon requires the presence of IL-2, and serves as a self-regulatory form of antigen-specific T cell depletion which contributes to down-regulation of the immune response following antigenic encounter.(Snow et al., 2010) T cells from patients with both SAP deficiency and XIAP deficiency demonstrate abnormal sensitivity to RICD. SAP is required for normal RICD to proceed, and ablation of SAP results in decreased upregulation of FasL and BIM (Bcl-2-interacting mediator of cell death) upon TCR restimulation and impaired apoptosis. (Snow et al., 2009). SAP may also influence cell death by interacting with the anti-apoptotic valosin-containing protein (VCP).(Nagy et al., 2009)

In sharp contrast, the opposite effect is observed in XIAP deficiency, and XIAP-deficient T cells possess an increased susceptibility to undergo RICD.(Rigaud et al., 2006; Marsh et al., 2010) The increased RICD observed in patients with XIAP deficiency can be more difficult to observe depending on the methodology used to induce RICD, which precludes it from being a practical screening assay for XIAP deficiency at this time.

The defect in RICD observed in SAP deficient patients can be readily observed in experienced laboratories (Figure 5). However, unlike the flow cytometric tests discussed above, evaluation of RICD is a cumbersome and time-intensive process. Peripheral blood mononuclear cells are first separated from whole blood by Ficoll-Hypaque density gradient centrifugation. T cells are then stimulated with mitogen or receptor-specific antibodies, followed by culture in the presence of exogenous IL-2, which is required for T cell expansion and acquisition of susceptibility to RICD.(Lenardo et al., 1999). Activated T cells are later plated in duplicate or triplicate and exposed to various concentrations of either plate-bound or soluble activating anti-CD3 antibodies. After adequate exposure, samples can then be stained with propidium iodide (or other suitable stains) and analyzed by flow cytometry. Cell death can be quantified as $\% \text{ cell loss} = (1 - (\% \text{ or absolute \# viable cells, treated} / \% \text{ or absolute number viable cells, untreated})) \times 100$ (Figure 5).

Conclusions

Here we have reviewed evidence that flow cytometry offers several tools which can be used to screen patients for SAP and XIAP deficiencies. Direct measurement of intracellular SAP and XIAP can offer a straightforward diagnosis of patients while awaiting results of genetic sequencing, and can also be used to study the impact of mutation upon protein expression. This can be especially helpful when previously undescribed missense mutations are encountered, as interpretation of genetic sequencing alone can be challenging in this situation.

Flow cytometry also offers the ability to identify bimodal expression of proteins, which allows diagnosis of female carrier status. It additionally offers a simple means of monitoring lineage-specific donor and recipient chimerism in the setting of hematopoietic cell transplantation.

Lastly, flow cytometry can be used to study patient iNKT cell populations and T cell susceptibility to RICD. These methods require an understanding of the difficulties that can be associated with rare event flow cytometry, and an appreciation of the time needed for the study of RICD. These issues limit the practicality of these studies for use as routine screening assays, but they can offer valuable adjunctive information when evaluating patients for XLP.

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Figure 1.

Flow cytometric detection of SAP in peripheral blood CD8+ T cells and NK cells from a normal control, a patient with SAP deficiency, and a maternal carrier. Whole blood lymphocytes were first surface stained with fluorochrome-conjugated antibodies against CD3, CD8, and CD56. IntraPrep reagent (Immunotech) was used for fixation and permeabilization according to the manufacturer's instructions. The cells were then incubated with the unconjugated rat anti-SAP monoclonal antibody KST-3,(Shinozaki et al., 2002) or rat IgG1, followed by FITC-labeled goat anti-rat IgG. After washing, cells were resuspended in 1% paraformaldehyde and analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). CD8+ T cells were defined as CD3+ and CD8+ lymphocytes. NK cells were defined as CD3- and CD56+ lymphocytes. The percentage positive staining for intracellular SAP was determined with comparison to the isotype-specific control antibody. The open black histograms represent the isotype control, and the colored open histograms represent SAP staining.

Figure 2.

Flow cytometric analysis of XIAP in peripheral blood leukocytes from a representative normal control, a patient with XIAP deficiency due to a nonsense mutation in BIRC4, and a patient with XIAP due to a missense mutation in BIRC4. Anticoagulated whole blood lymphocytes were first fixed and permeabilized using a commercially available kit (Intraprep, Beckman Coulter). Cells were then incubated with the anti-XIAP antibody, clone 48 (BD Biosciences). After washing, cells were incubated with PE-conjugated goat anti-mouse (BD Biosciences) secondary antibody. Samples were washed followed by blocking of residual PE-conjugated anti-mouse antibody with mouse IgG1 antibody (BD Biosciences) followed by standard fluorochrome-conjugated surface marker antibody staining using mouse anti-CD3-PerCP, -CD4-Pacific Blue, -CD19-FITC, and -CD56-APC (BD Biosciences). (Surface staining is performed after the permeabilization and XIAP staining to

prevent binding of the secondary PE-conjugated anti-mouse antibody to the murine surface marker antibodies). Samples were analyzed on a BD FACSCanto Flow Cytometer using FACSDIVA software, with data analysis using FCS Express (De Novo Software). After appropriate lymphocyte subset gating, the percentage positive staining for intracellular XIAP was determined with comparison to the isotype-specific control antibody. The open black histograms represent the isotype control, and the colored filled histograms represent XIAP staining.

Figure 3.

Flow cytometric analysis of XIAP in peripheral blood leukocytes from a patient with XIAP deficiency before allogeneic HCT, 28 days following allogeneic HCT, and 56 days following allogeneic HCT. N/A= not applicable, as there was no measurable B cell reconstitution at these time points. The open black histograms represent the isotype control, and the colored filled histograms represent XIAP staining.

Figure 4.

Flow cytometric analysis of iNKT cell populations of a representative normal control, a patient with SAP deficiency, and a patient with XIAP deficiency. Whole blood samples were incubated with fluorochrome-conjugated anti-CD3 (BD Biosciences), anti-TCRV α 24, and anti-TCRV β 11 (Beckman Coulter) monoclonal antibodies to identify iNKT cells. Erythrocytes were lysed by incubation in FACSlyse (BD Biosciences). Samples were washed, fixed, and analyzed on a BD FACSCanto flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences), with data analysis using FCS Express.

Figure 5.

A. Flow cytometric analysis of T cell RICD of a representative normal control and a patient with SAP deficiency. Patient or control PBMCs were first suspended at $0.5\text{--}1 \times 10^6$ cells/ml in RPMI 1640 media supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin/streptomycin (Invitrogen). T cells were activated with 5 mcg/ml Concanavalin A (Sigma). After 4 days, activated T cells were washed and then cultured in medium supplemented with 100U/ml recombinant human IL-2 for 18-23 days. Expanded T cells (1×10^5 cells/well) were then plated in duplicate in 96-well plates and treated with the anti-CD3 monoclonal antibody OKT3 (Ortho Biotech) (500 ng/ml). Twenty-two hours after treatment, cells were stained with propidium iodide and analyzed on a FACSCanto flow cytometer (BD Biosciences). Cell death was quantified as % cell loss = $(1 - (\% \text{ viable cells, treated} / \% \text{ viable cells, untreated})) \times 100$. B. Graphic representation of results of flow cytometric analysis of T cell RICD among normal controls and patients with SAP deficiency.