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[http://www.cincinnatichildrens.org/svc/prog/  
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## Patient Vignette (part 1 – Presentation)

The Clinical Immunology and Research Immunology Laboratories are integrated in a comprehensive Immunodeficiencies and Histiocytosis Program at Cincinnati Children's Hospital Medical Center (CCHMC). It interfaces with the Molecular Genetics Laboratory (<http://www.cincinnatichildrens.org/research/div/genetics>), as part of the Diagnostic Center for Heritable Immunodeficiencies, as well as with the Blood and Marrow Transplantation Program. To illustrate these interactions, each Newsletter will present a Patient Vignette, starting with an interesting result obtained in the Laboratory.

The figures on this page represent a SAP assay, i.e. flow cytometric detection of intracellular SAP (SLAM [signaling lymphocyte activation molecule] Associated Protein) in a patient (**left**) and healthy control (**right**). Top histograms are gated on CD8+ T-cells; middle on NK-cells; lower on NKT-cells. Green line represents isotype control; purple represents anti-sap staining.

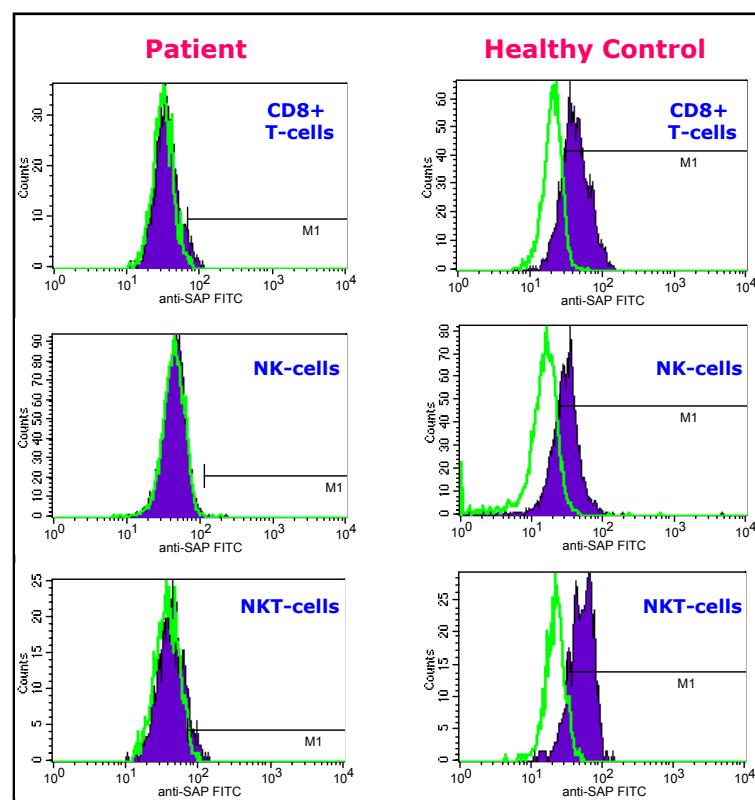
The results reveal a lack of SAP expression in all cytotoxic T-cells subsets.

The patient is a 6-year boy with infectious mononucleosis, caused by Epstein-Barr virus (EBV).

*See Part 2—Discussion on page 5*

EBV serology was consistent with acute EBV infection. In addition, highly elevated levels of EBV DNA were detected in his peripheral blood. Past medical history was unremarkable and the family history revealed one healthy older sister and a maternal uncle who had died from complications of lymphoma at the age of 15.

Other immunologic findings included increased immunoglobulins, the presence of atypical lymphocytes that showed an activated phenotype and highly elevated levels of soluble IL-2Ralpha 34195 pg/ml; normal range= 186-2678 pg/ml). Both NK and CTL function, as determined by *in vitro* NK- and CTL-assays, were significantly decreased.



## New Assays

The Diagnostic Immunology Laboratories, consisting of the Clinical Immunology Laboratory and the Research Immunology Laboratory, are committed to providing the highest quality, comprehensive clinical testing available to aid in the detection, diagnosis and treatment of pediatric immunologic, as well as oncologic and hematologic, disorders. We're committed to applying scientific advances to promote efficiency, enhance patient care and improve clinical utility.

The clinical diagnostic laboratories are in compliance with all major regulatory agencies including CLIA (Clinical Laboratory Improvement Amendments), CAP (College of American Pathologists), HCFA (Health Care Financing Administration), HIPAA (Health Insurance Portability and Accountability Act) and JCAHO (Joint Commission on Accreditation of Healthcare Organizations).

The current menu of immunologic assays and information regarding shipping instructions is published on the back page of this Newsletter. The accompanying [Test Requisition Form](#) can be obtained through our website at:

<http://www.cincinnatichildrens.org/svc/prog/>



Dan Marmer

Immunophenotyping of peripheral blood lymphocytes can provide useful information in patients with an immunodeficiency disorder, especially when placed in the context of functional measurements of the immune system. The flow cytometric **ALPS Panel** is intended to screen for the presence of Autoimmune Lymphoproliferative Syndrome (**ALPS**). Together with clinical and other laboratory data, the **ALPS Panel** allows preliminary determination as to whether the patient has **ALPS**. Further testing, including genetic testing and demonstration of defective *in vitro* FAS-mediated apoptosis may be required to complete the diagnostic workup.

### Clinical Introduction

**ALPS** is a primary immunodeficiency disorder (PID), characterized by defective lymphocyte homeostasis. Its main clinical manifestations are lymphoproliferation, causing lymphadenopathy, [hepato]splenomegaly and hypersplenism, autoimmunity (autoimmune cytopenias and other autoimmune disorders) and a highly increased, lifelong, risk of lymphoma. From a laboratory standpoint, there is defective FAS-mediated apoptosis, the presence of T-cells that lack CD4 and CD8 expression (so-called **α/β-DNTCs**), other immunophenotypic changes involving lymphocytes, hyperimmunoglobulinemia, elevated levels of interleukin (IL)-10 and vitamin B12, and the presence of auto-antibodies. **ALPS** is linked to genetic defects in the gene encoding FAS (**TNFRSF6**) in approximately 75% of patients (classified as **ALPS Ia**). In several other patients, defects have been found in the genes encoding FAS Ligand and caspase-10 (classified as **ALPS Ib and II**, respectively), while ~20-25% of patients remain without a genetic diagnosis (classified as **ALPS III**). Current diagnostic guidelines include: chronic non-malignant lymphadenopathy or splenomegaly, presence of **α/β-DNTCs** (>1% of total lymphocytes or >33 absolute number of cells) and defective FAS-mediated lymphocyte apoptosis *in vitro*.

### ALPS PANEL

#### Principle of the Assay

This flow cytometry-based assay evaluates 4 immunophenotypic parameters, commonly found to be abnormal in **ALPS** patients. Parameter # 1 determines the overall percentage of **α/β-DNTCs** [R2], while parameter # 2 determines the proportion of **α/β-DNTCs** that expresses B220 [R3]. Parameter # 3 measures two opposing T-cells effects, the ratio between CD3/CD25 [R4] and CD3/HLA-DR positive cells [R5]. In ALPS there is an increase in HLA-DR-positive cells (on **α/β-DNTCs** and CD8+ T-cells), with a concomitant loss/absence in CD25-positive T-cells (due to loss/absence of CD4/CD25 cells). This ratio is abnormal in patients with ALPS, while comparable to healthy controls in relatives, who share the **TNFRSF6** mutation, but do not have clinical ALPS. The 4th parameter measures the percentage of memory B-cells in the peripheral blood (defined by CD27 expression) [R6]. In **ALPS** patients, there is a reduced percentage of CD27+ B-cells.

Age-dependency is present for #2 and #4. Results are regarded in the context of an age-matched reference range. If the patient is taking medications, the results of this assay need to be regarded with caution. This particularly applies to immunomodulating agents (e.g. corticosteroids, rituximab).

#### Example of an ALPS Panel

A representative **ALPS Panel** is depicted on the next page, with a healthy control (**HC**) on the left, and a patient with **ALPS** on the right. The patient is a 14 year old male, who presented at the age of 5 years with chronic lymphadenopathy and splenomegaly. Autoimmunity affecting neutrophils and platelets has been present during the last 5 years, requiring intermittent therapy (steroids, high-dose IVIG, and rituximab). Lymphadenopathy has largely resolved; splenomegaly remains present. The patient has a mutation in **TNFRSF6**, affecting the intracellular death domain of Fas.

# From the Diagnostic Immunology Laboratories

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**ALPS is a prototypic primary immunodeficiency disorder of defective homeostasis of the immune system.**

The presence of T-cells that express the alpha/beta T-cell receptor, but lack both CD4 and CD8 ( $\alpha/\beta$ -double negative T-cells) is virtually pathognomonic for ALPS.

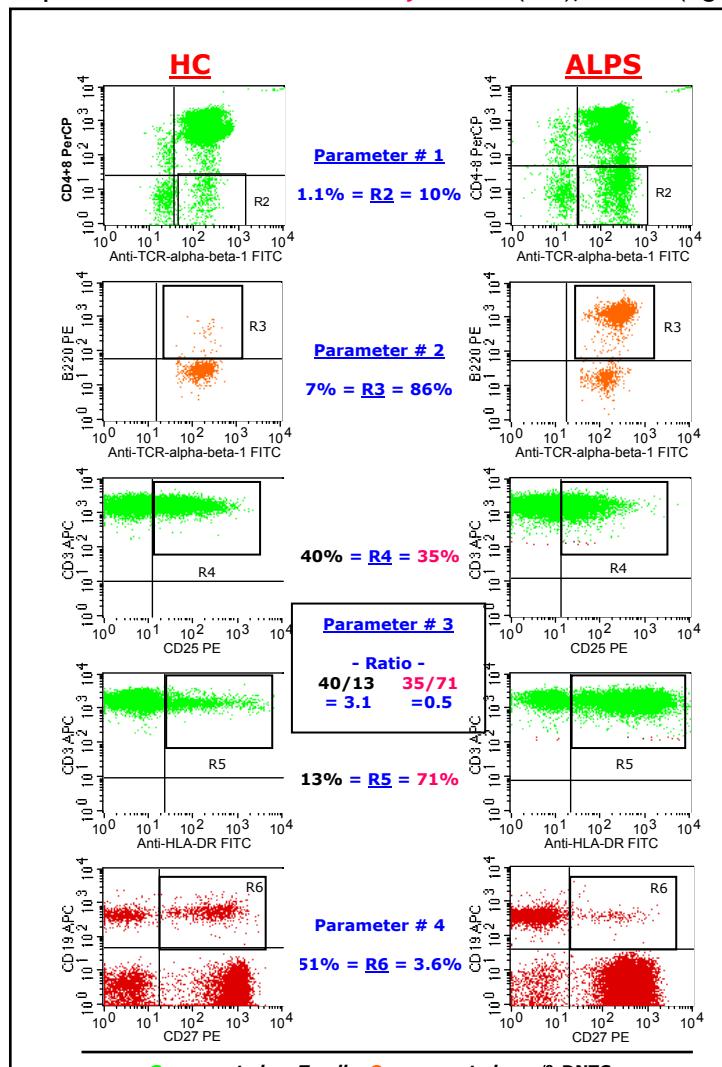


Kathryn Quinn

In the majority of patients, ALPS is inherited as an autosomal dominant disorder, due to mutations in the gene encoding FAS/CD95 (TNFRSF6).

The Immunodeficiency & Histiocytosis Group offers comprehensive programs for ALPS, including genetic testing and treatment programs.

Representative ALPS Panel. Healthy Control (left); Patient (right)



## Interpretation of Results

The first 2 parameters are unique for ALPS, reflect lymphoproliferation and indicate if this is on the basis of defective function of FAS (indicated by the expression of B220). Particularly in cases, in which there is a minimal expansion of  $\alpha/\beta$ -DNTCs (e.g. because the patient is on immunosuppressive medications), adding the B220 marker (with near 100% sensitivity and specificity) allows one to distinguish between an incidental finding of increased  $\alpha/\beta$ -DNTCs, and the pathognomonic expansion of B220-positive  $\alpha/\beta$ -DNTCs, seen only in ALPS patients.

The third and fourth parameters evaluate aspects of T- and B-cell dysfunction; possibly linked to autoimmunity. These immunophenotypic findings can also be found in disorders of immunodysregulation that share features with ALPS, such as common variable immunodeficiency and other immunodeficiency disorders.

Following detection of ALPS or suspected ALPS, further workup may be indicated, which involves laboratory testing (e.g. IL-10) and genetic testing of TNFRSF6 (<http://www.cincinnatichildrens.org/research/div/genetics>).

# From the Diagnostic Immunology Laboratories

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**Upregulation of CD64 on neutrophils is one of the earliest events in the inflammatory cascade.**

**This assay cannot be used in the absence of neutrophils. In addition, caution should be used in patients receiving therapeutic G-CSF, as G-CSF induces CD64 upregulation.**



Virgil Villanueva

**\*Research and Development are integral parts of the Clinical Immunology and Research Immunology Laboratories at CCHMC, and are conducted in close collaboration with the Immunodeficiencies and Histiocytosis Program.**

Following the release of pro-inflammatory cytokines and mediators (e.g. interferon-gamma [INF- $\gamma$ ] and granulocyte-colony stimulating factor (G-CSF)) in patients with new onset sepsis or tissue injury, there is a rapid increase in expression of CD64 on neutrophils. Thus, measurement of neutrophil CD64 up-regulation can provide useful and timely information regarding impending sepsis and tissue injury, complementing other data (e.g. CRP). CD64 is the high-affinity Fc receptor for IgG. Its (up-regulated) expression on neutrophils is measured by flow cytometry, using the Leuko64™ kit (Trillium Diagnostics, LLC).

The kit is composed of a mixture of monoclonal antibodies with specificities to CD64 and CD163 (monocyte marker) and a fluorescent bead suspension used for instrument calibration and to provide quantitative results for CD64 and CD163 expression (i.e. "standard curve").

## CD64 UPREGULATION

Constitutive expression of CD64 on monocytes (identified by size and CD163 expression) provides an internal positive control, while lack of CD64 on lymphocytes (identified by size) provides an internal negative control. In combination with automated software for data analysis, using cluster-finding algorithms, the kit has a rapid turn-around time for result reporting, making this an assay specifically intended for use in suspected sepsis. The results correlate well with CRP values.

Potential applications include: evaluation of sepsis, evaluation of fever in patients receiving medications that can cause fever (e.g. monoclonal antibodies), fever in transplant patients to distinguish between infection and rejection (solid organ transplantation) or graft-versus-host disease (bone marrow transplantation). Since neutrophils up-regulate CD64 in response to G-CSF, this assay can also be used to determine the response to exogenous G-CSF.

## Down the Pipeline\*

### PLASMA CYTOKINES

Cytokines are small, secreted, proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They are produced *de novo* in response to certain (immune) stimuli. They generally (although not always) act, over short distances and short time spans and at very low concentrations, by binding to specific membrane receptors, which then signal the cell, to alter its behavior. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. It is common for different cell types to secrete the same cytokine or for a single cytokine to act on several different cell types (pleiotropy). Cytokines are redundant in their activity, meaning similar functions can be stimulated by different cytokines.

The measurement of the levels of cytokines and/or soluble markers of immune activation can provide reliable information regarding the disease diagnosis, disease stage, prognosis, and response to therapy. Applications cover the entire medical spectrum, but are most relevant in conditions that are associated with immunodeficiency, inflammation and autoimmunity. For example, detecting and monitoring of pro-inflammatory cytokines can be used to predict and monitor response to disease modifiers (e.g. infliximab, anakinra) in the case of rheumatologic disease or inflammatory bowel disease. Isolated increased levels of IL-10 can be used in the diagnosis of ALPS.

## CYTOKINES INCLUDED IN PANEL

IL-1 $\beta$	IL-2
IL-4	IL-5
IL-6	IL-8
IL-10	INF- $\gamma$
GM-CSF	TNF- $\alpha$



Linda Poole / Dan Marmer

**XLP is a primary immunodeficiency disorder characterized by defective cytotoxic responses to EBV infection.**

For further information on XLP and references, visit GeneReviews at: <http://www.genetests.org/>

The Immunodeficiency & Histiocytosis Group offers comprehensive programs for XLP, including genetic testing, and bone marrow transplantation.

Cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically (two or more cytokines acting together) or antagonistically (cytokines causing opposing activities). The short half-life, low plasma concentrations, pleiotropy, and redundancy of cytokines all complicate their isolation and characterization. Cytokine levels in plasma and other biological fluids are now recognized as potential and useful markers of ongoing clinical disorders.

A new technology using spectrally encoded antibody-conjugated beads can be used to measure several different cytokines simultaneously. In general, Multiplex Bead Immunoassays represent solid phase sandwich immunoassays, which are read on specialized machines. By monitoring the spectral properties of the beads and the amount of fluorescence, the instrument measures the concentration of one or more analytes present in the original specimen.

The Plasma Cytokine Assay will be available, as a 10-cytokine panel in October, 2005.

## Patient Vignette (part 2 – Discussion)

The decreased SAP expression in all cytotoxic lymphocyte populations (CD8+ T-cells, NK-cells and NKT-cells), as detected by the flow cytometric SAP assay, together with the clinical history and laboratory testing (cytotoxicity assays) point towards X-linked lymphoproliferative disease (XLP). DNA was submitted to the Molecular Genetics Laboratory for mutational analysis of *SH2D1A*, the gene mutated in XLP. The result revealed a disease-causing mutation in *SH2D1A*. The mother and grandmother (the mother of the maternal uncle) were found to be carriers of this mutation, while the older sister was not.

XLP is a primary immunodeficiency disorder affecting (cytotoxic) lymphocyte function, especially, but not exclusively, in response to EBV. This particular family illustrates two possible clinical phenotypes, associated with *SH2D1A* mutations: 1] severe, fatal to near fatal, infectious mononucleosis. This is the most common presentation of XLP, occurring in >50% of patients; 2] Lymphoma and lymphoproliferative disease. These occur in about one-third of cases, some of whom have survived an initial EBV infection. The lymphomas seen in XLP are typically high-grade B-cell lymphomas, non-Hodgkin type, often extranodal, particularly involving the GI system.

Males, who develop a second distinct lymphoma (not relapse) after achieving initial remission after standard chemotherapy for lymphoma, should be suspected of XLP.

The other clinical phenotypes include hypogammaglobulinemia (~30% of patients), which can manifest itself as common variable immunodeficiency (CVID), and less frequently aplastic anemia, vasculitis, and lymphoid granulomatosis. Since SAP is required for normal cytotoxic function, EBV-infection may cause secondary/reactive hemophagocytic lymphohistiocytosis (HLH).

Mutations in *SH2D1A* are inherited in an X-linked manner. *De novo* mutations in *SH2D1A* are rarely identified; therefore, mothers of males with proven mutations in *SH2D1A* are very likely to be carriers. Prenatal diagnosis is possible in known carriers (who may demonstrate germ-line mosaicism). Genotype-phenotype relationships are not apparent. Large deletions do not cause a more severe phenotype, and considerable phenotype variability can exist within a family.

Allogeneic bone marrow transplantation (BMT) is the only curative therapy of XLP. Supportive treatment includes IVIG, rituximab, acyclovir, and others.



# From the Diagnostic Immunology Laboratories

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## IN THE NEXT ISSUE

Patient Vignette - A Case of Mistaken Immunologic Identity

The B-cell Panel - From Recent Bone Marrow Emigrant to Plasma-blast

CD163 - A Marker of Macrophage and Monocyte Activation

F-actin Polymerization - A New Look at Neutrophil Function



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[www.cincinnatichildrens.org/svc/prog/immunodeficiencies](http://www.cincinnatichildrens.org/svc/prog/immunodeficiencies)

### Test Requisition Form 2005

send samples at room temperature by priority overnight courier to:  
Julie Beach  
Cincinnati Children's Hospital Medical Center  
Hematology/Oncology CHRF 1301  
3333 Burnet Avenue, Cincinnati, OH 45229

Please call 513-636-4685 with tracking # prior to shipping

Patient Name _____	Date of Sample _____	Time of Sample _____
Medical Record # _____	Date of Birth _____	Sex: Male _____ Female _____
Race: African American _____ American Indian _____ Asian _____ Hispanic _____ White _____ Other (specify) _____		
Diagnosis or reason for testing _____	ICD9 code _____	
Please specify any family history _____	Is there consanguinity? yes _____ no _____	
Medications: _____	Recent or current viral infections: _____	
Disease status: active disease _____ remission _____	Has the patient undergone BMT? no _____ yes (date of BMT) _____	

Test(s) being requested: \*Note: please include results of a same day CBC/diff when ordering these tests

<input type="checkbox"/> *ALPS panel	CPT #88185x9	\$403	3ml (1ml) EDTA
<input type="checkbox"/> Antigen Stimulation	CPT #86353	\$444	10ml (5ml) Sodium Heparin
<input type="checkbox"/> *CD40L (CD154)	CPT #88184; 88185x3	\$156	5ml Sodium Heparin
<input type="checkbox"/> *CD45RA / CD45RO	CPT #88184; 88185x4	\$389	3ml (1ml) EDTA
<input type="checkbox"/> *CD59 (PNH Screen)	CPT #88184	\$85	3ml (1ml) EDTA *ON ICE*
<input type="checkbox"/> CD64 (Leuko64)	CPT #88184	\$48	1ml (0.5ml) EDTA
<input type="checkbox"/> *CD132 / CD127	CPT #88184; 88185x5	\$468	3ml (1ml) EDTA
<input type="checkbox"/> CTL function	CPT #86849	\$471	10ml (5ml) Sodium Heparin
<input type="checkbox"/> Cytokines, Intracellular (IL-4, IFN-γ, TNF-α)	CPT #88184; 88185x2	\$910	3ml (2ml) Sodium Heparin
<input type="checkbox"/> Cytokines, Plasma (panel of 10)	CPT #83520	\$1387	5ml (3ml) EDTA
<input type="checkbox"/> EBV immortalized cell line	CPT #86849	\$172	3ml Sodium Heparin
<input type="checkbox"/> *Lymphocyte Activation Markers	CPT #88184; 88185x8	\$559	5ml Sodium Heparin
<input type="checkbox"/> *Lymphocyte subset analysis (CD3,4,8,16/56,19) call for CPT codes		\$302	3ml (1ml) EDTA
<input type="checkbox"/> Mitogen Stimulation	CPT #86353	\$667	10ml (5ml) Sodium Heparin
<input type="checkbox"/> Neutrophil function battery			please call to schedule
<input type="checkbox"/> NK function	CPT #86849	\$377	10ml (5ml) Sodium Heparin
<input type="checkbox"/> Perforin/Granzyme B	CPT #88184	\$406	3ml (1ml) EDTA
<input type="checkbox"/> SAP protein (for R/O XLP)	CPT #88184	\$411	3ml (1ml) Sodium Heparin
<input type="checkbox"/> Soluble CD163	CPT #83520	\$512	3ml (1ml) EDTA or Na Hep
<input type="checkbox"/> Soluble IL-2R $\alpha$	CPT #83520	\$209	3ml (1ml) EDTA or Na Hep
<input type="checkbox"/> *TCR α/β / TCR γ/δ	CPT #88184; 88185x4	\$453	3ml EDTA
<input type="checkbox"/> *TCR V beta clonogram	CPT #88184; 88185x23	\$728	3ml EDTA
<input type="checkbox"/> *Wiskott-Aldrich Syndrome Protein (WAS)	CPT #88184	\$190	5ml Sodium Heparin
<input type="checkbox"/> ZAP70	CPT # 88184	\$218	3ml EDTA or NaHep
<input type="checkbox"/> Other _____			



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