



NEW ASSAYS

GM-CSF Receptor Stimulation and GM-CSF Auto-Antibody Assays to Aid in the Diagnosis of Pulmonary Alveolar Proteinosis (PAP)

Pulmonary alveolar proteinosis is a rare syndrome characterized by impaired pulmonary surfactant clearance from the alveolar space that can lead to respiratory failure, and by innate immune deficiency involving macrophage- and neutrophil-mediated host defense. Clinically, PAP is diagnosed in the presence of a compatible history, typical radiologic findings, and lung biopsy or bronchoalveolar lavage findings. However, these methods do not identify the underlying PAP-causing disease responsible.

PAP-causing diseases can be grouped into three categories: primary, secondary, and congenital. Primary PAP includes autoimmune PAP and hereditary PAP, which are caused by an autoantibody to GM-CSF (GMAB) or mutations in the genes encoding the GM-CSF receptor (*CSF2RA/B*) that cause disease by disrupting GM-CSF signaling. Secondary PAP is associated with a variety of hematological malignancies, various environmental exposures, infections, or states of immune deficiency. Congenital PAP is caused by mutations in genes required for normal surfactant production. According to Dr. Bruce Trapnell, autoimmune PAP accounts for 85% of cases.

STAT5 is an important protein in the GM-CSF signal transduction pathway and is activated upon phosphorylation of tyrosine. Patients with primary PAP (hereditary or autoimmune) will exhibit decreased phosphorylation of STAT5 due to the mutations in GM-CSF receptor or the presence of high levels of auto-antibodies to the cytokine, respectively. The DIL recently adapted assays developed in the laboratory of Dr. Trapnell to aid in the diagnosis of patients with hereditary and autoimmune PAP. These assays can also be used when GM-CSF receptor mutations or GM-CSF autoantibodies are suspected in patients with certain fungal infections. (Continue reading next page.)

IN THIS ISSUE:

New Assays

GM-CSF Receptor Stimulation	1-2
Anti-GM-CSF Auto-Antibody.....	2-3
T-cell Degranulation.....	3-4
Hemoglobin-Oxygen Affinity, p50	5
Osmotic Gradient Ektacytometry	5-6

Bulletin Board.....	7
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In Upcoming Issues	7
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GM-CSF Receptor Stimulation Assay (DIL)

GM-CSF is added in differing concentrations to whole blood and incubated at 37°C. The cells are then fixed, lysed, permeabilized, and stained with antibodies against phosphorylated STAT5 (pSTAT5). The cells are then analyzed on a flow cytometer. Normal control cells will contain phosphorylated STAT5 even at lower levels of GM-CSF. Cells from Autoimmune PAP patients will typically only phosphorylate STAT5 at higher concentrations of GM-CSF, due to the interference of autoantibodies. Hereditary PAP patients will typically express no increased phosphorylation of STAT5 at any GM-CSF concentration, due to the mutations in the GM-CSF receptor. See Figure 1 and 2 for examples of the analysis on a healthy adult control as well as a patient with autoimmune PAP.

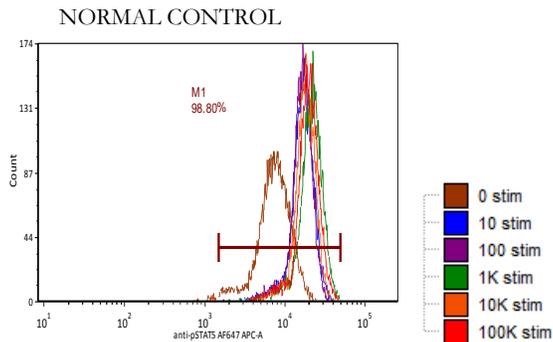


Figure 1: The cells are first classified on the flow cytometer using CD45 and side light scatter. Once the neutrophils have been identified, these cells are assessed for pSTAT5 staining. The M1 gate is adjusted to include all the overlays and the GM-CSF Receptor Stimulation Index is calculated. In this case, phosphorylation has occurred across all concentrations of GM-CSF indicating no GM-CSF autoantibody presence or GM-CSF receptor mutation.

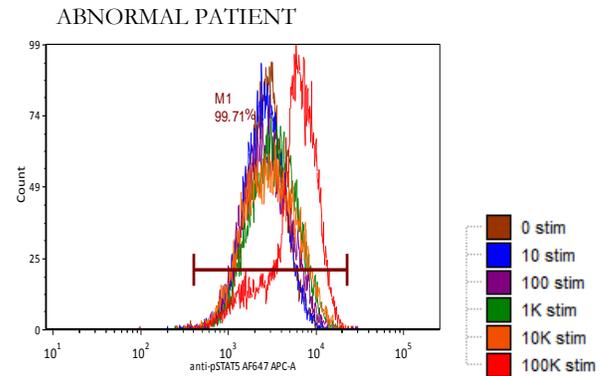


Figure 2: In this case, phosphorylation has occurred only at the 100K level indicating the patient may have autoimmune PAP. The presence of autoantibodies does not allow the PMNs to initiate phosphorylation at the lower levels of GM-CSF stimulation. This will result in a lower GM-CSF Receptor Stimulation Index.

Anti-GM-CSF Antibody Assay (GMAb Assay) (DIL)

An ELISA for anti-GM-CSF antibodies utilizes a human recombinant GM-CSF antigen as a capture protein to coat the wells of a microtiter plate. Standards and the serum of patients or healthy individuals are appropriately diluted and added to the wells after blocking and washing. Next, a goat anti-human IgG labeled with horseradish peroxidase is used to detect the antigen-antibody complex attached to the well. The wells are washed and a substrate solution is added. Hydrolysis of the substrate by peroxidase produces a color change. The reaction is stopped by adding an acid stop solution and the color changes are measured. A standard curve is constructed and the patient or healthy individual autoantibody concentrations are measured. Normal pediatric and adult controls do not produce high levels of GM-CSF autoantibodies (Figure 3).

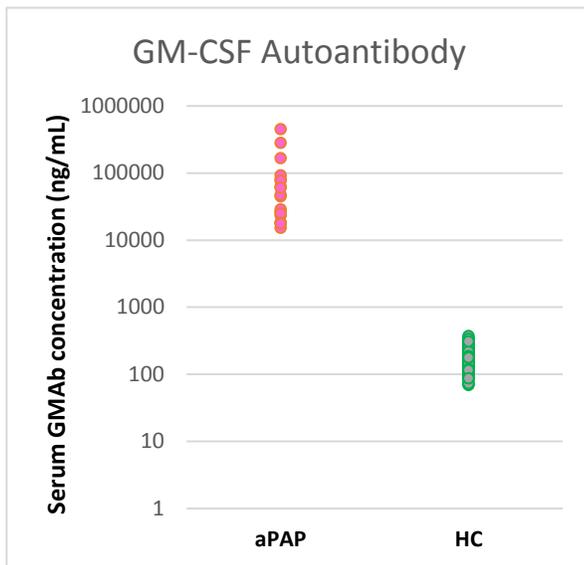


Figure 3: Normal and abnormal GMAb concentrations expressed in ng/ml. Serum GMAb concentration in 19 autoimmune PAP patients (aPAP) and 75 healthy normal controls (HC) was measured using the Anti-GM-CSF Antibody Assay.

References:

1. Uchida, K et al. (2014) Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. *J Immunol Methods*. 402(1-2):57-70.
2. Uchida, K et al. (2004) High-affinity autoantibodies specifically eliminate granulocyte-macrophage colony stimulating factor activity in the lungs of patients with idiopathic pulmonary alveolar proteinosis. *Blood*; 103: 1089-1098

Aknowledgements and Further Information

Both the GM-CSF Receptor Stimulation Assay and the Anti-GM-CSF Antibody Assay were validated with the assistance of Dr. Bruce C. Trapnell, MD, Brenna Carey, PhD, and Claudia Chalk, RAIV.

For information regarding levels of GMAb typically observed in patients with autoimmune PAP, see Uchida, K et al. (2014) Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. *J Immunol Methods*. 402(1-2):57-70.

T-Cell Degranulation (DIL)

Evaluation of T-cell degranulation via measurement of CD107a can be used as a diagnostic screening test for genetic hemophagocytic lymphohistiocytosis (HLH) due to mutations in UNC13D, STX11, STXBP2, RAB27A, and LYST. CD107a, also known as LAMP-1 (Lysosome-associated membrane protein – 1), is normally expressed on the membranes of lytic granules contained within cytotoxic CD8+ T cells and NK cells. These granules contain perforin, granzymes, and other cytotoxic contents. When cytotoxic CD8+ T cells are presented with a target cell, these granules are mobilized to the cell membrane where they fuse with the membrane and release their contents. This whole process is called degranulation. As this happens, CD107a is transiently expressed on the effector cell surface.

In this assay, mononuclear cells from peripheral blood samples are exposed to P815 cells coated with anti-CD3 (the P815 cell line is a murine mastocytoma which readily binds the Fc portions of the anti-CD3 antibody), which stimulates degranulation. PE-conjugated anti-CD107a is present during the stimulation period. After a 2 hour incubation at 37°C, the cells are surface stained with markers to allow the analysis of T cells. If the T cells have degranulated, the CD107a will be expressed on the effector cell surface and will be detected by flow cytometry. See Figure 1 and 2 for examples of the analysis on a healthy adult control and a patient with STXBP2 mutations. T- cell degranulation may be particularly helpful for diagnostic testing of patients with suspected genetic HLH but low numbers of NK cells which prevents NK cell CD107a testing to be performed.

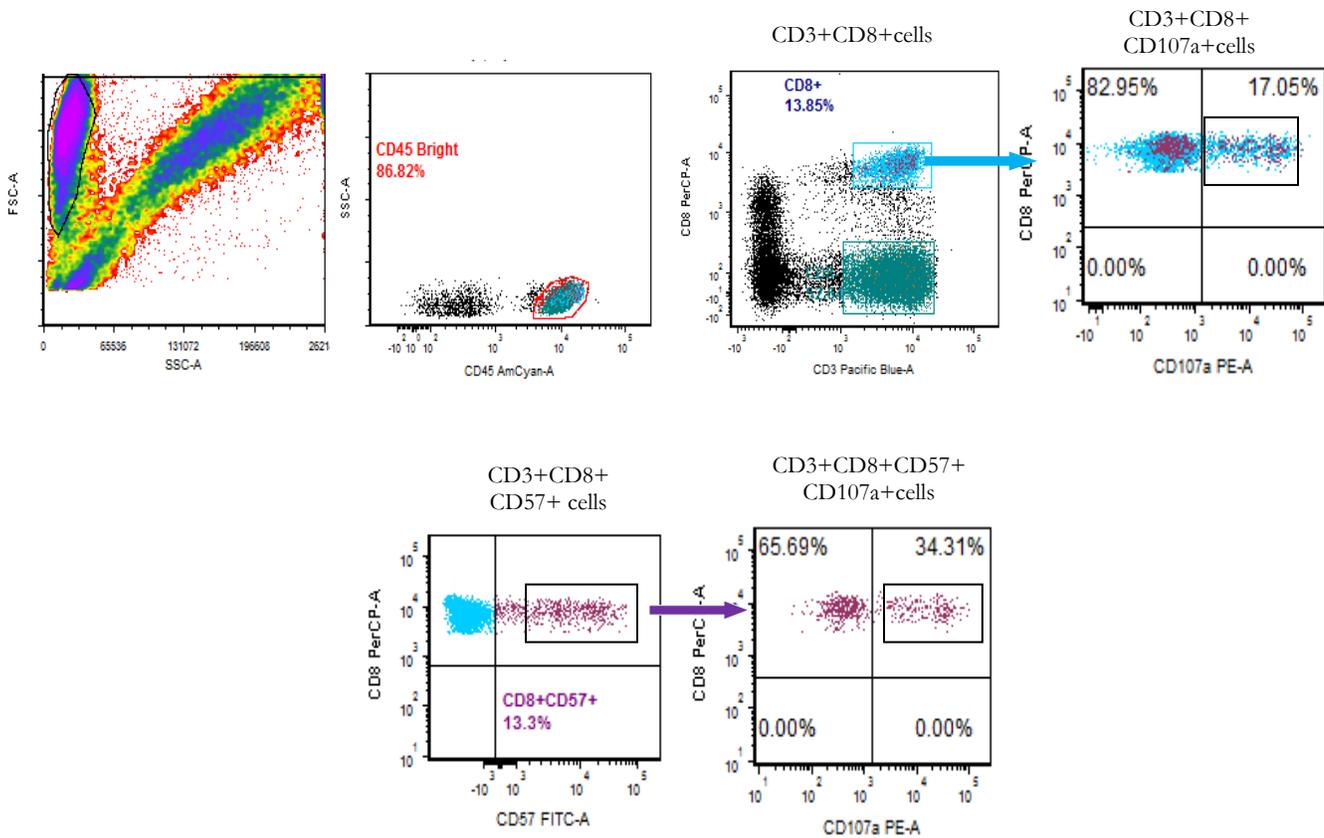


Figure 1: Healthy adult showing normal surface expression of CD107a. Cytotoxic T lymphocytes are isolated using a combination of side light scatter, bright CD45, CD3 and CD8 staining patterns. CD107a expression is calculated as a percentage on two cell populations, CD3+CD8+ cells and CD3+CD8+CD57+ cells. The normal range calculated from the 5/95th percentile is 1.4% to 35.2% and 10.0% to 65.3% respectively.

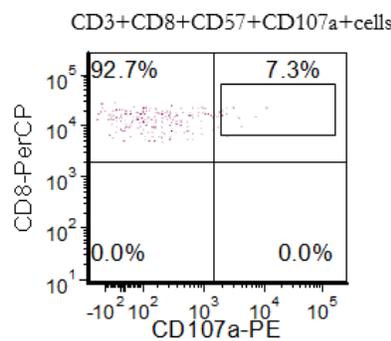


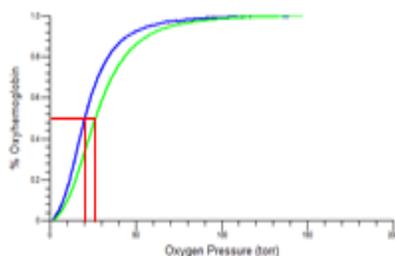
Figure 2. Histogram showing 107a expression on CD3+CD8+CD57+ cells of a patient with an STXBP2 mutation. The expression of 7.3% is well below normal.

Hemoglobin-Oxygen Affinity, p50 (EDL)

The CCHMC CBDI Erythrocyte Diagnostic Laboratory (EDL) is now offering an assay to determine the oxygen affinity of hemoglobin (i.e., measurement of the oxygen-hemoglobin dissociation curve).

This test can aid in the diagnosis of inherited conditions that affect hemoglobin oxygen affinity (e.g., hemoglobinopathies and other intrinsic RBC abnormalities), which may present with anemia, polycythemia, or cyanosis. This test can also help to narrow the differential diagnosis of acquired forms of anemia, polycythemia, or cyanosis, some causes of which might be associated with abnormal oxygen affinity (e.g., carboxyhemoglobinemia or methemoglobinemia).

This assay uses the Hemox-Analyzer (TCS Scientific), with an operating principle based on dual-wavelength spectrophotometry for the measurement of the optical properties of hemoglobin and a Clark electrode for measuring the oxygen partial pressure. The results of this assay are reported as the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen (the p50). The reference range for p50 is 23.47 – 28.48 mmHg. A low p50 indicates increased oxygen affinity. A high p50 indicates decreased oxygen affinity. Below is an example of an oxygen dissociation curve for a normal vs abnormal (high oxygen affinity):



- Normal Control
- High O2 Affinity, p50 = 20.4 mmHg

Osmotic Gradient Ektactometry (EDL)

Ektactometry measures RBC deformability under a defined shear stress as a function of suspending medium osmolality. The test is used to evaluate for inherited RBC membrane disorders, which are commonly responsible for hemolytic anemia, differentiating between hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP), Southeast Asian ovalocytosis (SAO), and hereditary stomatocytosis (HSt).

Ektactometry is recommended as the next step after hemoglobin electrophoresis in the diagnosis of a non-immune hemolytic anemia. An abnormal ektactometry profile confirms a red cell membrane disorder and assists with the differential diagnosis between hereditary spherocytosis versus elliptocytosis/pyropoikilocytosis versus xerocytosis. This is particularly important since in xerocytosis splenectomy is contraindicated because it precipitously increases the risk for life-threatening thrombophilia. Ektactometry in a patient with a non-transfusion dependent hemolytic anemia may save the need to perform a hereditary hemolytic anemia genetic panel or focus the genetic work-up to a red cell membrane panel if ektactometry is positive or to a red cell enzyme panel if ektactometry is negative.

The test is performed using the Osmoscan LoRRca MaxSis (Mechatronics Instruments BV®, Zwaag, The Netherlands). Three distinct features of the osmotic gradient ektactometry profiles are: **i) Omin:** corresponds to the value of the hypotonic osmolality where 50% of the cells hemolyze in an osmotic fragility assay and provides information on the initial surface to volume ratio of the cell sample.

ii) **EImax**: Elongation Index reaches a maximum near 300 mOsm/kg, suggesting that the normal red cell deforms optimally at the tonicity to which it is normally exposed. The value of EImax depends mostly on the cytoskeleton mechanics. iii) **Ohyp**: The declining portion of the curve (the osmolality value where the cells are at half of the maximum elongation) correlates with the initial MCHC (intracellular viscosity) of the cell sample (see Figure 1). The Ektacytometry patient report contains an ektacytometry profile (patient versus a normal control), the Omin, EImax, and Ohyp values and an interpretation. Examples of RBC membrane disorders detected by Ektacytometry can be seen in Figure 2.

For more information regarding either Hemoglobin-Oxygen Affinity or Osmotic Gradient Ektacytometry, please visit the CBDI EDL website (www.cchmc.org/EDL), or call (513)636-4234.

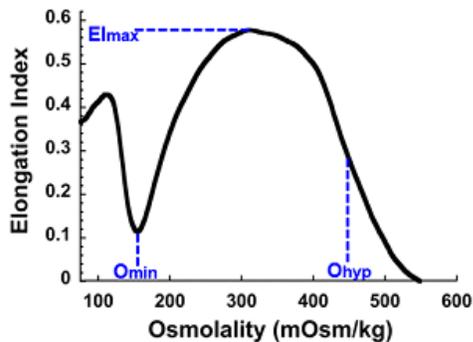


Figure 1: The Ektacytometry Curve

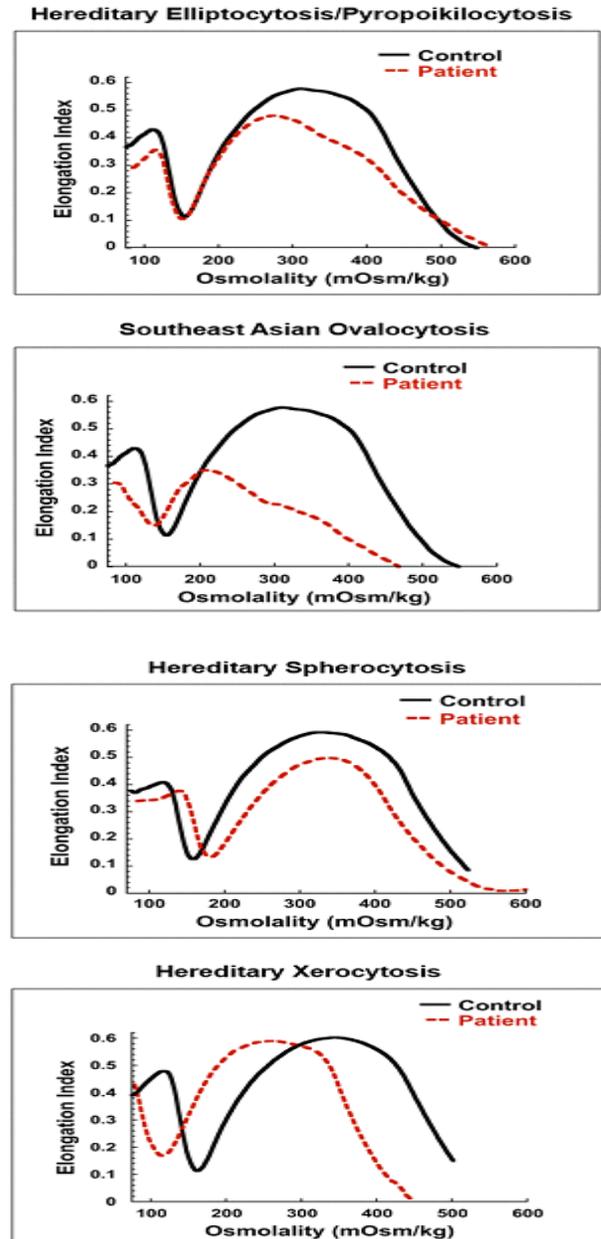


Figure 2: Examples of red blood cell membrane disorders detected by Ektacytometry

References:

1. Mohandas N, Clark MR, Jacobs MS, Shobet SB. Analysis of factors regulating erythrocyte deformability. *J Clin Invest.* 1980; 66(3):563-573.
2. Da Costa L, Sumer L, Galimand J, et al. Diagnostic tool for red blood cell membrane disorders: Assessment of a new generation ektacytometer. *Blood Cells Mol Dis.* 2016; 56(1):9-22.

The CBDI Clinical Laboratories consist of the Diagnostic Immunology Lab (**DIL**), the Erythrocyte Diagnostic Lab (**EDL**), the Immunopathology Lab (**IPL**) and the Hemostasis and Thrombosis Lab (**HTL**). We are committed to providing the highest quality testing available to aid in the diagnosis and treatment of pediatric immunologic, hematologic, and oncologic disorders.

The CBDI labs are in compliance with all major regulatory agencies including CLIA, CAP, HCFA, HIPAA, and JCAHO.

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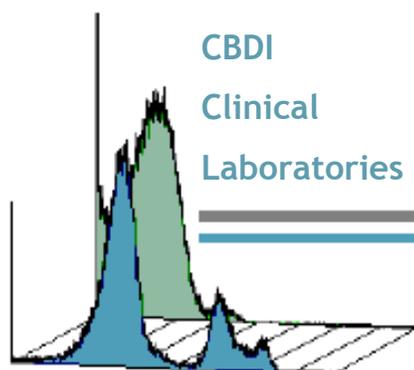
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BULLETIN BOARD:

Spring Meeting

Please stop by and say “hello” in April at the American Society of Pediatric Hematology/Oncology (ASPHO) meeting in Montreal this year. We look forward to seeing you in person and meeting new faces, as well! We will be there as the Cincinnati Children’s Clinical Laboratories.

QNS (Quantity Not Sufficient) Specimens

Many of our functional tests require a minimum number of lymphocytes (as opposed to a minimum blood volume) in order to perform the assay, as noted on our requisition. We have developed a chart that can give you an estimate of the volume of blood needed for these assays based on your patient’s absolute lymphocyte count (ALC) obtained from a CBC/Diff. To view this chart, please visit the website or click to open our [Customized Volume Chart](#)

Feedback

We like to hear from our Clients. We invite you to share your questions and comments with us. Feel free to call or email your feedback to CBDILabs@cchmc.org

IN UPCOMING ISSUES:

Alemtuzumab Levels
CXCL9 -*now clinically available*
S100 Proteins Panel