

From the Diagnostic Immunology Laboratories

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Spring Meeting

Please stop by and say “hello” in April at this year’s American Society of Pediatric Hematology/Oncology (ASPHO) Annual Meeting in Miami, Florida. We will be there as the Cincinnati Children’s Clinical Laboratories with the Molecular Genetics and Nephrology Laboratories.



The Laboratory Staff at one of our monthly Continuing Education Presentations

THIS ISSUE'S FOCUS – B Cell Panel

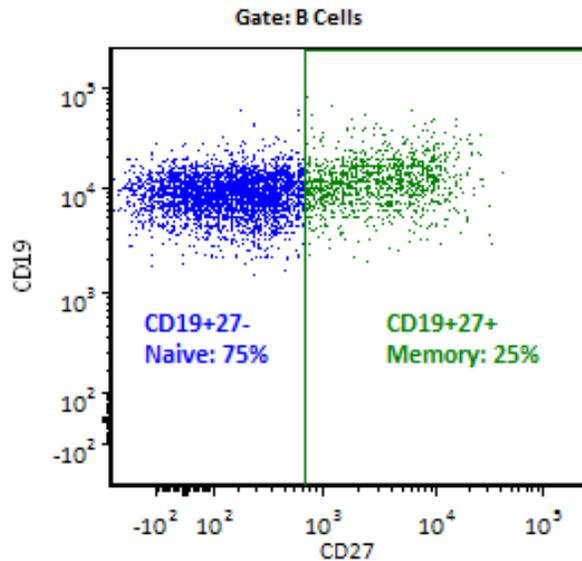
We are very pleased to officially introduce our revised B cell panel. The assay was modified to make better use of expanding multiparameter capabilities and to include several new immunophenotypic markers.

It is important to gain an overview of the major B cell subsets and identify alterations in B cell differentiation associated with immune mediated disorders. The most recent update to the B cell studies in the DIL, involves several aspects of B cell maturation and differentiation as defined in the following description of the new panel.



Section I – Total B cells & Naïve vs. Memory:

Total B cells are quantified with CD19. CD27 vs. CD19 allows us to establish how many naïve (CD27-) and memory (CD27+) cells there are. CD19 is also compared with CD20 expression for verification of total B cells and appropriate marking of maturity.



Section II – Immature B cells:

Immature B cells are quantified to provide an estimation of recent Bone Marrow emigrants. Recent BM emigrants and immature B cells, in general, will be CD19+CD10+ and reflect immature B cells prior to the stage of transitional B cells.

Section III – Transitional B cells:

In this section of the panel, we look strictly at the CD19+CD27- B cells. We evaluate the transitional populations for CD10, CD21, CD38, and IgM.

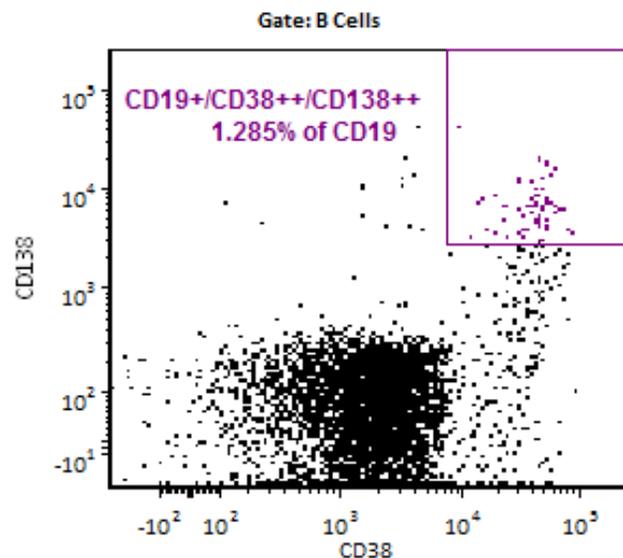
Section IV – Mature, Naïve B cells:

Mature, naïve B cells are CD27 negative, CD21 positive, and IgM and IgD positive. The cells we observe in this part of the panel are once again CD19+CD27-. From this combination, we can establish the presence of a population of CD21-CD38- which is important in CVID classification. IgM+IgD+ naïve cells are normal, and the lack of the two surface immunoglobulins indicates isotype-switched cells (IgM-IgD-).

Section V – Memory B cells: Memory B cell populations stain as CD19+CD27+. These cells can be divided into non-isotype-switched memory B cells, IgM+/IgD+, and isotype-switched memory B cells, which are IgM-IgD-.

Section VI – CD5 Expression: It is believed that CD5 is found on the B-1a subset of B cells (producing mostly low-affinity polyreactive IgM antibodies that recognize a variety of self and foreign antigens) and not expressed on the B-1b or B2 subset. CD5 upregulation on B cells plays a role in tolerance to auto-antigens. CD5 has also been implicated in identifying which B cells are activated. Lastly, CD5 expression can be used to assess the process of immune reconstitution, following B cell depleting therapy (e.g. rituximab).

Section VII – Plasmablasts/Plasmacells: Plasma blasts/cells will be CD38++ and CD138++. Activated circulating B cells, as well as terminally differentiated plasma-blasts and early plasma cells, can be identified on the basis of CD38 and CD138 expression and down-regulation of CD19 expression (CD20 expression is already lost at this stage).



What's the same:

- Analysis of CD19, CD5, CD27, B220
- Plasmablasts/cells.
- Age-dependent reference ranges (Figure 1 below shows changing CD5 and CD27 expression on B Cells)

What's New:

- Analysis of CD20 on CD19+ B cells.
- Addition of IgM and IgD to distinguish between non-switched and switched memory cells (and to verify the predominantly non-switched nature of naïve B cells, lacking CD27)
- We are able to provide a much more detailed analysis of the subsets in the transitional and naïve B cell populations to reconcile patients' clinical presentations with their underlying problems and direct treatment and care decisions.

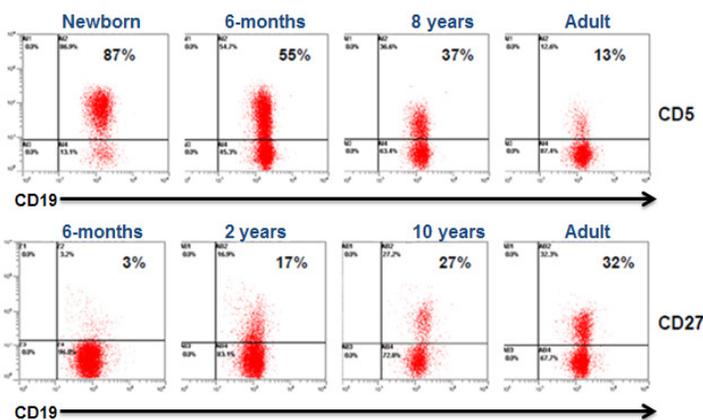


Figure 1. CD5 and CD27 expression of B cells change with advancing age: CD5 expression decreasing; CD27 expression increasing.

Patient Application:

An adult patient with decreased IgG/IgA and increased IgM who was non-responsive to vaccines showed a pattern with very elevated CD19+27-21-38- (“CVID population”) of 6.8% as well as demonstrating an absence of any class-switched memory B cells (Figure 2). Patients with CVID that have a markedly reduced number of switched memory B cells are at risk of having a more severe clinical phenotype.

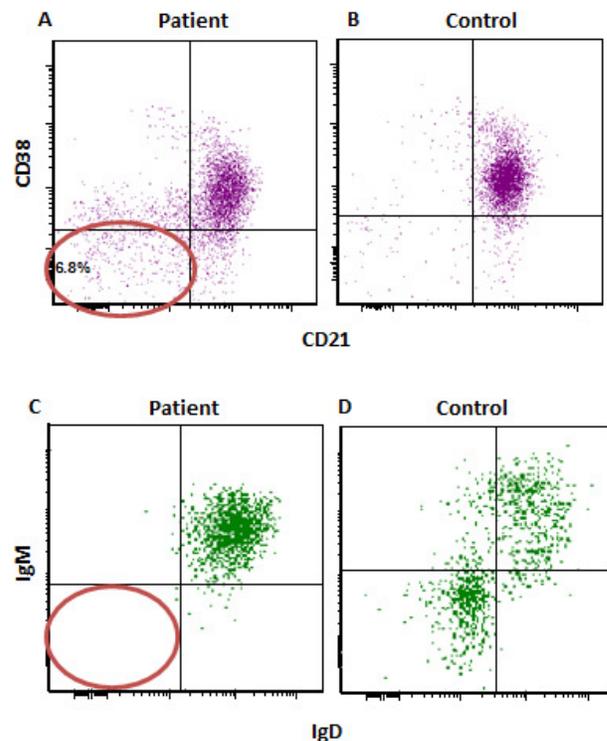


Figure 2. A patient sample (A) an adult control (B). A and B are taken from the CD19+CD27- B cell gate. The patient (A) shows an elevated CD21-38- (“CVID” population) of 6.8% of cells gated. Observe the adult control (B) with very few of these events. The same patient and control are shown in C and C from the CD19+CD27+ gate and the patient (C) shows an absence of any class-switched memory B cells (CD19+27+IgM-IgD-). D is provided to show an example of a healthy adult.

UPDATE ON LABORATORY ASSAYS FOR ALPS INVESTIGATIONS

Autoimmune Lymphoproliferative Syndrome is primarily a defect in the normal apoptosis of lymphocytes. Since the expected programmed death of cells does not take place, there is lymphoproliferation and resulting autoimmunity.

Diagnostic criteria for ALPS, as decided by the 2009 NIH International Workshop, are divided into 2 required criteria and 6 accessory criteria. Diagnosis of ALPS is based on both required criteria being present and at least one of the accessory criteria.

Required:

- Chronic nonmalignant, noninfectious lymphadenopathy/splenomegaly
- Elevated CD3+TCRab+CD4-CD8- cells

Accessory:

- Defective lymphocyte apoptosis (in 2 separate assays)
- Increased biomarkers: Vit. B12, soluble FASL, IL-10 and IL-18
- Autoimmune cytopenias and elevated polyclonal IgG
- Family history of a nonmalignant/noninfectious lymphoproliferation
- ALPS immunohistologic findings
- Somatic or germline pathogenic mutation in *FAS*, *FASLG*, or *CASP10*

Elevation in cellular and biomarker components are considered relevant at the following levels:

- CD3+TCRab+CD4-CD8- (double-negative T cells, or DNTC) should be greater than 1.5% of total lymphocytes or 2.5% of CD3+ lymphocytes in the setting of normal or elevated lymph count.
- plasma sFASL ≥ 200 pg/mL
- plasma IL-10 levels > 20 pg/mL plasma Vitamin B₁₂ levels > 1500 ng/L
- plasma IL-18 levels > 500 pg/mL

Note that even though an increased presence of circulating TCRab+DNTC is a hallmark of this disease, initial identification with a lymphocyte subset should be confirmed with a panel which includes other markers seen in ALPS such as the CD3/CD25 relationship to CD3/HLA-DR, numbers of memory (CD27+) B cells and the presence of B220 on the DNTC.

ALPS is indicated if between one and four of the following criteria are met:

- more than 2% of lymphs and/or 68 cells/mcL are $\alpha\beta$ +DNTC
- if more than 60% of $\alpha\beta$ +DNTCs express B220
- CD3/CD25 to CD3/HLA-DR ratio is less than 1
- Percentage of CD27+ B-cells less than 16%

It is also important to note that the presence of greater than 1.5% DNTC as a percentage of total lymphocytes is interpreted in the setting of a normal or elevated lymphocyte count. If there is a cytopenia, it is no longer as suggestive of an abnormality. This is because there may be an effect in cytopenias, especially lymphopenias, on the relative distribution of DNTC.

Elevations of DNTC above 3% of total lymphocytes and 5% of T lymphocytes are very indicative of ALPS. Patients with germline *FAS* mutations will usually have abnormal apoptosis results, but germline *FASLG* mutations and somatic *FAS* mutations will usually have normal apoptosis assay results. For this reason, it may be a time saver to order an apoptosis assay followed by an RICD extension so all three possibilities can be evaluated.

A Restimulation Induced Cell Death (RICD) is an extension of the apoptosis assay which evaluates TCR induced apoptosis of activated cells. These cells are effector memory T cells and are a Fas-sensitive subset of PBMC which are more resistant to apoptosis if a *FASL* or somatic *FAS* mutation is present.

Even though somatic *FAS* mutation is considered to be a more common cause of ALPS than either *FASLG* or *CASP10* mutations, it is sometimes difficult obtaining enough cells to perform the somatic *FAS* mutational analysis.

The **somatic *FAS* mutational analysis** is performed by sorting out DNTC from whole blood. This requires a large amount of blood in order to recover a relatively rare population, even in patients who have an increased percentage of DNTC.

- The sort is performed on BD FACS Aria Cell Sorters in the Flow Core Facility at Cincinnati Children's Hospital. The sort is scheduled and there is usually up to a two week wait for scheduled times.
- Diagnostic Immunology Laboratory staff performs the cells staining in preparation for the sort by tagging PBMC with CD3-PerCP and CD4, CD8 and CD56-FITC. The sorting then separates the CD3 positive cells that are neither CD4, CD8 nor NK cells.
- The DNTC population is submitted to the Molecular Genetics laboratory which requires around 500,000 cells in order to secure enough DNA for the *FAS* analysis.

The following is an example of how much blood is needed for the analysis:

- **If DNTCs are 3.2%** of the **total lymphocytes** and the patient has an **ALC of 1000 cells/mcL**, the **DNTC absolute** is 32 cells/mcL, or 32,000 cells/mL of blood
- In order to secure 500,000 cells with a 60% recovery from the sorter, approximately **30mL** of blood will be needed. A lower count would require more blood.

SUGGESTED ORDER OF TESTING:

1. Lymphocyte Subset analysis
2. ALPS Panel by flow cytometry
3. Apoptosis with RICD
4. Biomarker Vitamin B12
5. Biomarkers: IL-10 (presently available as part of DIL plasma cytokine panel), IL-18 (optional) and sFasL (sFasL and IL-10 will soon be orderable in DIL as an ALPS biomarker panel)
6. *FAS* mutational analysis (available in CCHMC Molecular Genetics)
7. Somatic *FAS* mutational analysis (available only if there is a success probability of sufficient cell recovery). Call Joyce V. at 513-636-8657 to discuss scheduling and blood requirements.

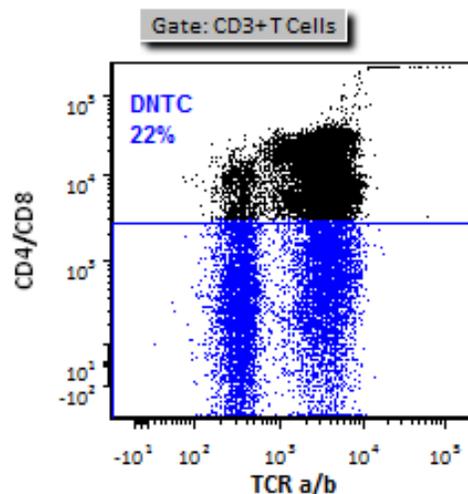


Figure 3. ALPS patient showing a very elevated DNTC population of 22% of total CD3 T Cells.

References:

Oliveira JB, Bleesing JJ, et al., Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010;116(14):e35-40.

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 last page of this Newsletter. The accompanying Test Requisition Form can be obtained through our website. Previous editions of the Newsletter can also be found at this website: www.cchmc.org/DIL

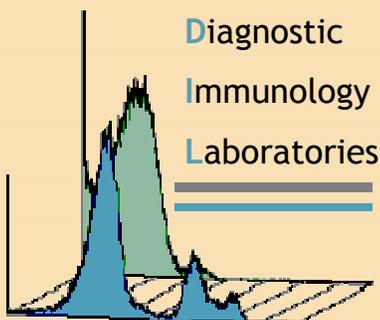
CONTACT US

Please visit our website or call us with any inquiries:

Ph: 513-636-4685

Fax: 513-636-3861

www.cchmc.org/DIL



New Tests Now Available:

- Revised B Cell Panel
- Revised CD45 RARO (Naïve/Memory) cell panel

Tests Coming Soon:

- ALPS Biomarkers: soluble FAS-ligand (sFASL), IL-10, and IL-18
- Th-17
- CD46 (for MCP mutations)

Feedback:

We would like to hear from our Clients. We invite you to share your questions and comments with us. Feel free to send/fax/email your comments to us: Fax 513-636-3861; Email: immunodeficiencies@cchmc.org



Artist rendering of the planned Seacrest Studios broadcast media center - the sixth in the country - intended to help lift the spirits of sick kids. The studio will stream live music, radio broadcasts, video and interactive games onto TVs in patient rooms. Construction begins in April 2013.

NEW Fillable Requisition on the Website:



DIAGNOSTIC IMMUNOLOGY LABORATORY

Phone: 513.636.4685 Please call with tracking information
Fax: 513.636.3861 - cchmc.org/DIL
Samples not accepted weekends

Ship First Overnight to:
CCHMC - Julie Beach
DIL - Rm R2328
3333 Burnet Avenue
Cincinnati, OH 45229-3039

DIL - TEST REQUISITION FORM

TESTS MUST BE RECEIVED MONDAY – FRIDAY WITHIN 24 HOURS OF BEING DRAWN

PATIENT INFORMATION

Patient Name (Last, First) _____, _____ Date of Birth: ____/____/____
 Patient Medical Record Number: _____ Date of Sample: ____/____/____ Time of Sample: _____
 Gender: Male Female Has the patient undergone a BMT? Yes No Date of BMT ____/____/____ Diagnosis Code: _____
 Race: African American American Indian Asian Caucasian Hispanic Other (Please Specify): _____
 Diagnosis or reason for testing: _____
 Medications: _____

TESTS REQUESTED – Recommended Volume (Minimum Volume)

SHIP UNSPUN AT AMBIENT TEMPERATURE UNLESS OTHERWISE STATED

<input type="checkbox"/> ALPS Panel	3ml (1ml) EDTA	CBC/Diff ²	<input type="checkbox"/> Neutrophil Adhesion Markers	3ml (1ml) EDTA
<input type="checkbox"/> Antigen Stimulation	10ml Sodium Heparin ¹		<input type="checkbox"/> Neutrophil Oxidative Burst	3ml (1ml) EDTA
<input type="checkbox"/> Apoptosis	20ml (10ml) ACD-A		<input type="checkbox"/> NK Function	10ml Sodium Heparin ¹
Note: Sample must be <24 hrs old and received Thursday AM				
<input type="checkbox"/> B Cell Panel	3ml (1ml) EDTA	CBC/Diff ²	<input type="checkbox"/> Perforin/Granzyme B	3ml (1ml) EDTA
<input type="checkbox"/> BAFF	3ml (1ml) EDTA ⁴		<input type="checkbox"/> PNH Screen (FLAER/CD59)	3ml (1ml) EDTA
<input type="checkbox"/> CD40L / ICOS	5ml (3ml) Sodium Heparin		<input type="checkbox"/> pSTAT5	3ml (1ml) EDTA
<input type="checkbox"/> CD45RA/RO	3ml (1ml) EDTA		<input type="checkbox"/> SAP (XLP1)	3ml (1ml) Sodium Heparin
<input type="checkbox"/> CD52 Expression	3ml (1ml) EDTA		<input type="checkbox"/> Soluble CD163	2ml (1ml) EDTA ⁴
<input type="checkbox"/> CD64 (Leuko64)	1ml (0.5ml) EDTA		<input type="checkbox"/> Soluble IL-2R	3ml (1ml) EDTA ⁴
<input type="checkbox"/> CD107a	10ml Sodium Heparin ¹		<input type="checkbox"/> Sorted Engraftment	Call to Schedule
Note: CD107 not accepted on Fridays or the day before a holiday				
<input type="checkbox"/> CD127/CD132	3ml (1ml) EDTA	CBC/Diff ²	<input type="checkbox"/> TCR α/β TCR γ/δ	3ml (1ml) EDTA
<input type="checkbox"/> CTL Function	10ml Sodium Heparin ¹		<input type="checkbox"/> TCR V Beta Repertoire	3ml (2ml) EDTA
<input type="checkbox"/> Cytokines, Intracellular	3ml (2ml) Sodium Heparin		<input type="checkbox"/> WASP	3ml (1ml) Sodium Heparin
<input type="checkbox"/> Cytokines, Plasma or CSF	5ml (3ml) EDTA ⁴ or CSF ³		<input type="checkbox"/> WASP Transplant Monitor	3ml (1ml) Sodium Heparin
<input type="checkbox"/> EBV Immortalized Cell Line	3ml Sodium Heparin		<input type="checkbox"/> XIAP (XLP2)	3ml (1ml) EDTA
<input type="checkbox"/> Check if EBV is a research sample; signed consent required				
<input type="checkbox"/> Foxp3	3ml (1ml) EDTA	CBC/Diff ²	<input type="checkbox"/> ZAP-70 (only for SCID)	3ml (1ml) EDTA
<input type="checkbox"/> INKT	3ml (1ml) EDTA		<input type="checkbox"/> Other _____	
<input type="checkbox"/> Lymph Activation Markers	3ml (2ml) Sodium Heparin		HLH - Recommended Tests	
<input type="checkbox"/> Lymphocyte Subsets	3ml (1ml) EDTA		Recommended for suspected HLH in order of priority:	
<input type="checkbox"/> MHC Class I & II	3ml (1ml) EDTA		<input type="checkbox"/> NK Function	10ml (5ml) Sodium Heparin ¹
<input type="checkbox"/> Mitogen Stimulation	10ml Sodium Heparin ¹		<input type="checkbox"/> Soluble IL-2R	3ml (1ml) EDTA ⁴
<input type="checkbox"/> Neopterin	3ml (1ml) EDTA ⁴ or CSF ³		<input type="checkbox"/> Perforin/Granzyme B	3ml (1ml) EDTA
			<input type="checkbox"/> CD107a	10ml (5ml) Sodium Heparin ¹
			<input type="checkbox"/> SAP (if patient is male)	3ml (1ml) Sodium Heparin
			<input type="checkbox"/> XIAP (if patient is male)	3ml (1ml) EDTA

IN THE UPCOMING ISSUE:

- TH-17

