From the Diagnostic Immunology Laboratories

Clinical Immunology Laboratory | Research Immunology Laboratory

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Susan Lee

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.
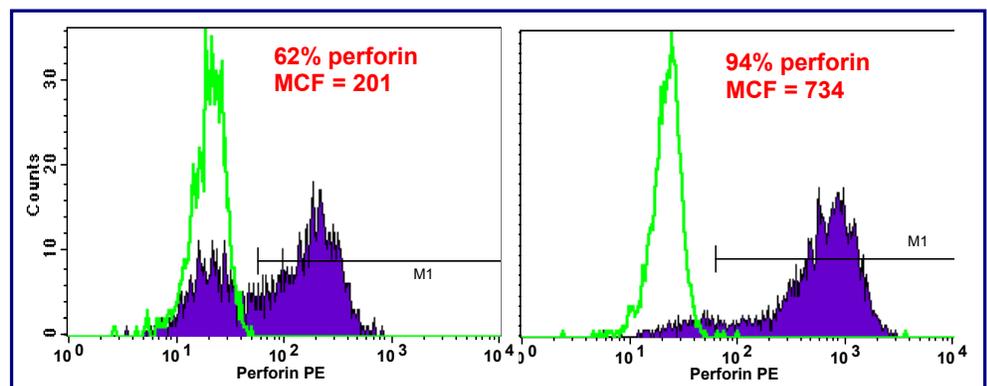
The patient is a 4-year boy who presented in the first year of life with a history of enteropathy and failure to thrive. Subsequently, he developed recurrent episodes of cellulitis, lymph node abscesses, and bacteremia. An immune work up at the time that included testing for chronic granulomatous disease, was reported to have been normal.

Although the patient did not have a history that was suggestive of a hemophagocytic lymphohistiocytic (HLH) disorder, perforin and granzyme B expression were measured during a recent immunologic workup to determine the presence of (unregulated) immune activation. The figures on this page represent flow cytometric detection of intracellular perforin in NK cells in the patient (left) and healthy control (right).

The green line represents isotype control; purple represents anti-perforin staining. The results reveal decreased, but not absent expression in NK cells, 62% in the patient versus 94% in the control, and a decreased level of mean channel fluorescence (MCF), 201 versus 734 (note: expression was low in cytotoxic T cells as well).

Our workup revealed, in addition to reduced perforin, reduced NK-cell function, normal lymphocyte enumeration (normal numbers of T, NK and B cells), and normal proliferative response to mitogens. Immunoglobulin levels showed normal IgG and IgM, but elevated IgA. Titers to tetanus, diphtheria and pneumococcus were reduced. Phenotyping of B cells showed normal numbers of B cells, but a significantly reduced memory B-cell compartment (see **fig. 3** on page 4).

62% perforin
MCF = 201

94% perforin
MCF = 734

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/ immunodeficiencies

**Darryl Hake
Joyce Villanueva**

New Assays

B-CELL PANEL

The study of B-cell development and differentiation is highly relevant for a better understanding of human disorders in which B cells are involved. These entail disorders characterized by recurrent infections (defective B-cell function), autoimmune disorders (inappropriate B-cell function) and B-cell malignancies. In many instances, the immunologic form (phenotype) of B cells that are involved in the disease process can be pathogenically linked to phases of B-cell differentiation, spanning the entire lifespan of the B cell, from the early B-cell precursor in the bone marrow to the terminally differentiated plasma cell. With advances in flow cytometry, for example with regard to the repertoire of monoclonal antibodies and the capacity for multiparameter analysis, clinical tools are now available to study the B-cell compartment in greater detail.

Principle of the Assay
The flow cytometric B-cell panel has been designed to provide a cost-effective overview of the major B-cell subsets and to identify key alterations in B-cell differentiation that are associated with immune-mediated disorders. Although projected to be run mostly on peripheral blood, it can be used (and adapted) for other relevant parts of the B-cell compartment (bone marrow, lymph nodes).

Following enumeration of total B cells, specific panels of monoclonal antibodies are used that identify B-cell subsets, reflective of differentiation from immature B cells (recent bone marrow emigrants, identified by CD10 expression), through transitional stages (captured by differential CD21 and CD23 expression) into mature B cells. Mature B cells (CD5 positive or negative), are divided into naïve B cells, characterized by negative CD27 expression (and positive B220 expression), and memory B cells, characterized by CD27 expression (and subdivided into B220-positive and B220-negative memory B cells). CD40 expression is included to screen for HIGM3 (caused by mutations in CD40), as a CD19/CD20-independent B-cell marker and as a B-cell activation marker (showing CD40 down-regulation). CD38 and CD138 expression are used to identify early plasmablasts and plasma cells that have started to down-regulate CD19 (as has been seen in patients with autoimmune disease), and as alternative markers of B-cell activation.

Upon identification of specific abnormalities, additional B-cell studies can be arranged, for example to further characterize immature and transitional B cells and memory B cell subsets (e.g. non-isotype switched versus isotype switched), as well as to evaluate the BAFF system.

Figure 1
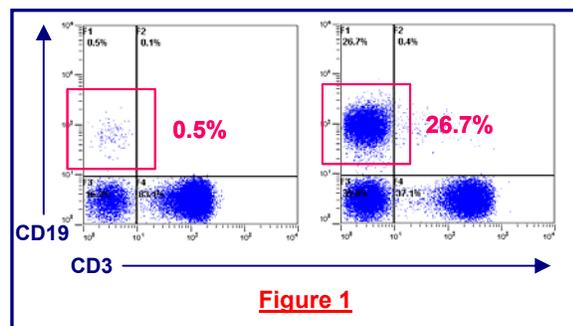
In the following section, examples will be shown to illustrate potential applications of this panel.

Basic enumeration of total B cells can assist in the diagnosis of immunodeficiency disorders, characterized by absent or low B-cell counts (for example X-linked agammaglobulinemia [XLA]).

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Genetic defects to consider in CVID:

BTK (causes XLA — males only)

SH2D1A (causes XLP — males only)

TACI (also linked to IgA deficiency)

ICOS

B-cell enumeration is helpful to classify patients with (suspected) common variable immunodeficiency (CVID, **fig.1**, previous page). CVID patients with B cells, but typically lacking memory B cells (see **fig. 3**) often have a different clinical phenotype than CVID patients with few or no B cells, particularly with respect to autoimmune, inflammatory and lymphoproliferative manifestations.

CD10 expression of peripheral blood B cells is typically low, but can be increased in patients with immunodeficiency disorders that affect the dynamics of B-cell generation and in patients, who recently received anti-B cell depletion therapy (e.g. rituximab).
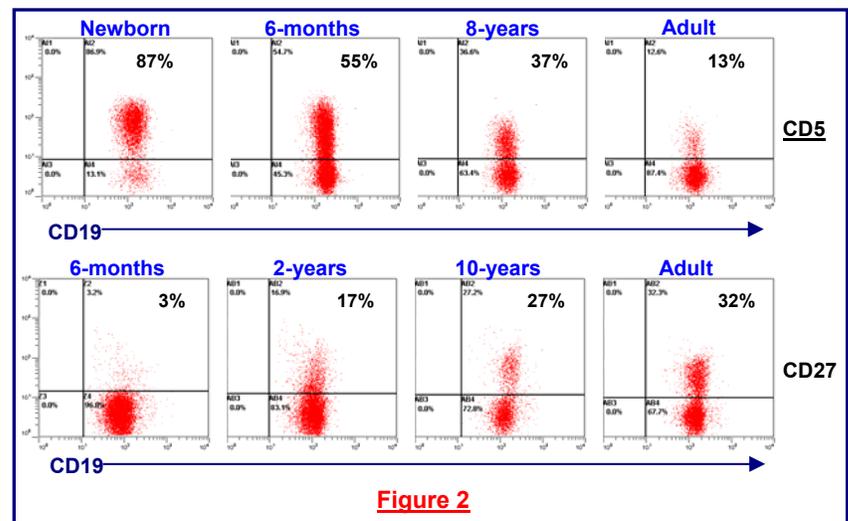

Figure 2

Patricia Atkins
Kathryn Quinn

As with many immunologic parameters, B-cell phenotypes should be regarded in the context of age. For example, as shown in **fig. 2**, CD5 and CD27 expression change with advancing age: CD5 expression decreasing; CD27 expression increasing. Age-related changes may be relevant for any B-cell marker of interest. Several age-matched control groups have been established for this purpose. These cover newborns through adults, and provide reference ranges to be used in the interpretation of data.

CD5 expression on B cells may have different implications in different situations. In certain scenarios, an increased number of CD5-positive B cells may be indicative of an expansion of a defined B-cell subset (analogous to the B1-type B cell in mice), while in other scenarios, CD5 may reflect activation.

A major advance in the clinical evaluation of B-cell integrity has been the discovery that peripheral blood B cells that have acquired a memory B-cell phenotype express CD27. Enumeration and characterization of memory B cells provides an image of overall B-cell competence as related to the germinal center reaction.

Although it cannot replace more traditional methods of evaluation of B-cell function, measuring memory B cells complements other studies of B-cell function and is particularly useful in patients in whom antibody responses to infection or vaccination cannot be (reliably) measured, such as patients receiving IVIG. In some of these individuals, finding a normal memory B-cell compartment may help in the decision to discontinue IVIG (permanently or temporarily to complete vaccination studies).

B-cell Panel, further reading:

1. Ferry et al. Clin Exp Immunol. 2005;140:532.

2. Ko et al. Clin Immunol. 2005;116:37.

3. Bleesing and Fleisher. Cytometry. 2003;51:1.

4. Noordzij et al. Pediatr Res. 2002;51:159.

PID with reduced memory B cells:

- CVID
- XLP
- HIGM
- LAD
- NEMO
- CGD
- WAS
- XLA
- [S]CID
- HIES
- DGS
- ALPS

Carol Moore

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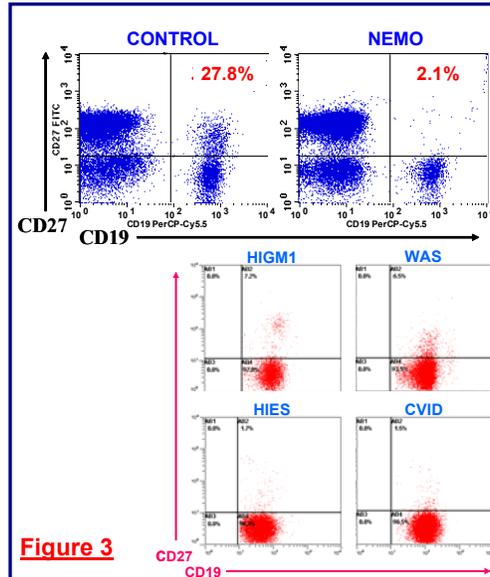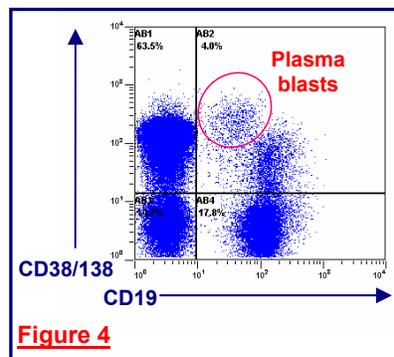Yan Zhong
Daniel Marmer
Julie Beach

Figure 3

Figure 4

There are many primary immunodeficiency disorders (PID) with reduced or absent memory B cells (see side box). In **fig. 3**, several examples are shown. At the top, CD27-positive B cells in a healthy control (left) and the patient described in the vignette on page 1 (right). Plots are gated on total lymphocytes, to show the (normal) presence of CD27 on T cells (left upper quadrants). Lower dotplots are gated on total B cells and were obtained from patients with X-linked hyper IgM symdrome (HIGM1), Wiskott-Aldrich syndrome (WAS), hyper IgE syndrome (HIES) and CVID.

At the other, final, end of B-cell differentiation are plasmablasts and plasma cells. These cells lose or down-regulate many B-cell markers (e.g. the B-cell receptor, CD19 and CD20), while up-regulating or expressing new markers, such as CD38 and CD138 (syndecan-1). Terminally differentiated plasma cells are typically not found in peripheral blood, but peripheral blood plasmablasts can be identified on the basis of CD19 down-regulation and high CD38/CD138 expression in certain conditions(see **fig. 4**, depicting a patient with systemic lupus).

It is the integrated interpretation of the immunophenotype, in combination with other data that allows concepts regarding the B-cell compartment to be developed and tested. Abnormal results need to be verified and reconciled with other data. The B-cell panel provides an assessment of B-cell phenotype that likely reflects its function; normal or abnormal.

ICOS UP-REGULATION

As mentioned, one of the applications of the B-cell panel is to distinguish between CVID patients with B cells and patients without (normal numbers of) B cells. In the latter group, several patients have been identified with autosomal recessive defects in the ICOS (inducible costimulator) gene. Similar to patients with the X-linked form of hyper-IgM syndrome (HIGM1), the primary defect in ICOS affects T cell activation and function.

Thus, CVID is theorized to be the result of defective interaction between activated (helper) T cells and B cells (expressing ICOS Ligand) during crucial stages of the germinal center reaction (B-cell proliferation, isotype switching, affinity maturation, as well as differentiation (and survival) of plasma cells and memory B-cells). Although CD40L defects appear to have consequences for T-cell function as well, this is less clear for ICOS deficiency

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Studies suggest that ICOS up-regulation on activated T cells can be used as a screen for ICOS-deficient CVID. It has been observed that the kinetics of ICOS up-regulation approximates that of CD40L (CD154) up-regulation, making it possible to combine ICOS and CD40L up-regulation in a single assay.

Although no ICOS-deficient CVID patients have so far been detected by the ICOS assay (approximately 5% of CVID patients are believed to be due to autosomal recessive defects in ICOS), interesting patterns have become apparent. Our first series of – abnormal - results appear to show two disparate CD40L/ICOS patterns.

In patients with HIGM1, abnormal CD40L up-regulation is accompanied with normal ICOS up-regulation, showing a selective defect in T-cell activation. In other patients, who do not have HIGM1 (following genetic evaluation of CD40L), there appears to be defective ICOS up-regulation in parallel with defective CD40L up-regulation, suggesting a more global problem in costimulation. Lastly, as shown in the figure below, and first reported by Ma et al in 2005 (JCI 2005;115:1049), patients with XLP appear to show isolated defective up-regulation of ICOS. This is of interest because XLP patients are known to have CVID-phenotypes, independent of EBV infection (see also the first DIL Newsletter).

Sue Vergamini
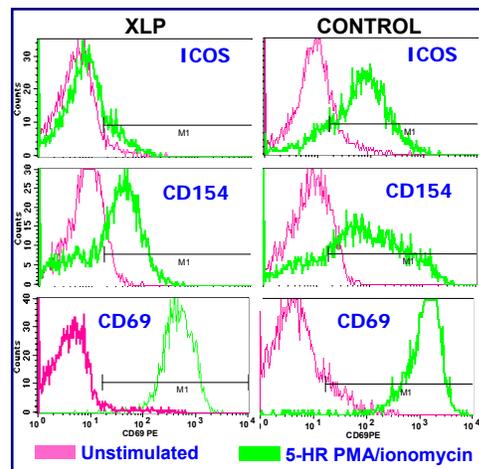
Down the Pipeline*

FOXP3 EXPRESSION

By suppressing both proliferation and cytokine production of auto-reactive T cells, T-regulatory cells play crucial roles in maintaining immunologic tolerance and preventing autoimmunity. Although there are multiple types of regulatory cells, one subset is defined, in part, by high expression of CD25 (the alpha chain of the IL-2 receptor) on their surface; designated $CD4^+CD25^{bright}$ T-regulatory cells (Tregs).

Further characterization of Tregs indicates that intracellular presence of the transcription factor FoxP3 (forkhead box P3, scurfin) is one of the most accurate markers identifying Tregs.
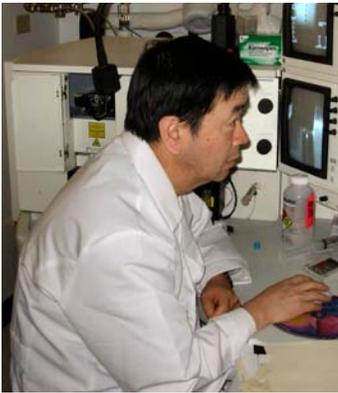
A central role of FoxP3 in the life of Tregs, is underscored by inherited defects of FoxP3. In humans, mutations in the FoxP3 gene are linked to the primary immunodeficiency disorder, IPEX (immunodeficiency, polyendocrinopathy, and enteropathy, X-linked).

Yan Zhong

IPEX/FOXP3, further reading:

1. Chatila. J Allergy Clin Immunol. 2005;116:949

2. Taams et al. Curr Top Microbiol Immunol. 2005;293:115.

3. Ochs et al. Immunol Rev. 2005;203:156.

4. Wildin et al. J Med Genet. 2002;39:537.

The predominating, and best characterized, IPEX phenotype is defined by generalized autoimmunity that starts in the first year of life. Autoimmunity involves insulin-dependent diabetes mellitus, enteropathy, and less frequently autoimmune cytopenias, autoimmune thyroid disease and tubular nephropathy. Enteropathy causes chronic diarrhea with severe failure to thrive. Dermatitis (eczematous, erythroderma) is present in many patients. Most males die within the first couple of years of life.

The diagnosis of IPEX is based upon clinical findings, in combination with identification of appropriate auto-antibodies. In about 60% of patients, the diagnosis is genetically confirmed by finding mutations in the FoxP3 gene. Medical management of IPEX includes immunosuppressive therapy (e.g. cyclosporine, tacrolimus) treatment of autoimmunity (e.g. insulin), nutritional support, and anti-microbial treatment and/or prophylaxis. Only bone marrow transplantation provides curative treatment.

Immunologic studies in IPEX are, in general, of limited value, because they are typically-normal, as they do not home in on the pathogenic aspects of IPEX.

If abnormal, it is often difficult to distinguish form the effects of immunosuppressive therapy. Recently, it has become possible to assay intracellular FoxP3 protein by flow cytometry. Our approach is to stain with an anti-FoxP3 antibody in combination with CD25, to co-localize FoxP3 relative to CD25 expression, and with CTLA-4 (cytotoxic T-lymphocyte associated protein 4, another immunophenotypic marker of Tregs). In the figure below, and example is shown of FoxP3 expression in a healthy adult, as well as a healthy infant (middle), compared to a 2 year old IPEX patient. Despite the fact that infants (and children) have different CD25-expression profiles than adults, both healthy individuals have relatively comparable levels of intracellular FoxP3, while the patient shows reduced (but not entirely absent) FoxP3 staining.

IPEX represents a condition in which Tregs are decreased/defective as a direct result of a genetic mutation in FoxP3. Other scenarios can be envisioned in which Tregs are compromised. We are focusing on several scenarios that have in common complex and/or combined autoimmune manifestations (e.g. autoimmune cytopenias in PID).
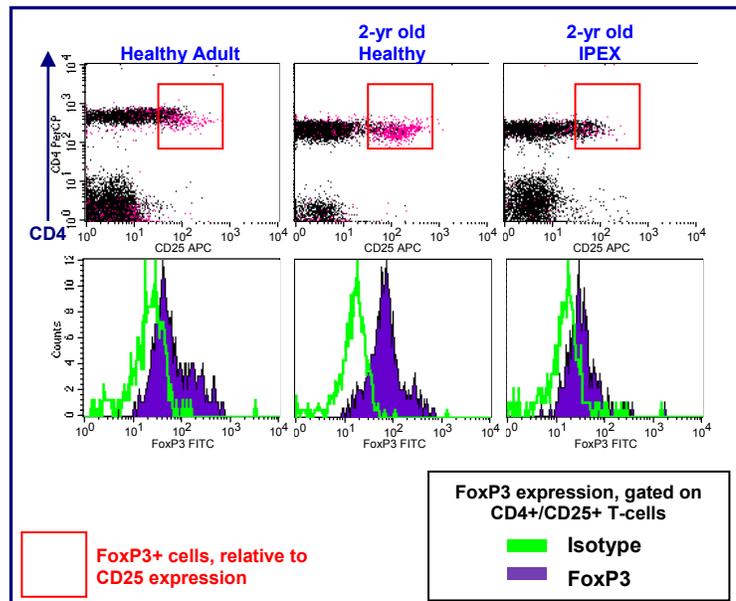

FoxP3+ cells, relative to CD25 expression

FoxP3 expression, gated on CD4+/CD25+ T-cells

Isotype
FoxP3

Anne Prada

NEMO, further reading:

1. Uzel G. Curr Opin Allergy Clin Immunol. 2005;5:513-8.

2. Puel A, et al. Curr Opin Immunol. 2004;16:34-41.

3. Orange JS, et al. J Allergy Clin Immunol. 2004;113:725-33.

4. Orange JS, Geha RS. J Clin Invest. 2003;112:983-5.

Patient Vignette (part 2 — Discussion)

Despite the reduced perforin expression, genetic analysis of the perforin gene was normal. As mentioned, the clinical history, albeit complex, was not consistent with a hemophagocytic lymphohistiocytic (HLH) disorder.

This case represents an interesting observation made in laboratory immunology: by performing a large number of assays in patients with both typical and atypical presentations of immunodeficiency disorders, other – often equally interesting – profiles become apparent. Very low to absent expression of perforin is highly suggestive of a primary (inherited) form of HLH, due to mutations in the perforin gene (PRF1). Heterozygote carriers of PRF1 mutations typically have perforin expression that is significantly lower than healthy controls (but higher than affected homozygotes/compound heterozygotes).

The absence of genetic alterations in perforin suggests that the similar pattern of perforin expression observed in our patient has a different basis. Detailed history and physical examination facilitated connecting the dots in this particular case. Review of systems revealed delayed dentition and an inability to sweat. Family history revealed several male relatives on the mother's side of the family who died in early childhood, presumably due to recurrent infections. Physical exam was notable for sparse hair, and a reduced number of teeth that were conical in shape. Based on these findings, a tentative diagnosis of primary immunodeficiency on the basis of an genetic defect in NEMO (nuclear factor kB essential modulator) was made, and subsequently confirmed by genetic testing.

Nuclear factor kB (NF-kB) plays a central role in the transcription of genes, involved in immunity, inflammation, apoptosis, and cell growth. NEMO is required to allow NF-kB to dissociate from its regulatory cytoplasmic subunit, migrate to the nucleus and initiate transcription. Loss-of-function mutations of NEMO cause X-linked dominant incontinentia pigmenti in affected girls, whereas in boys, they usually are lethal in utero.

Hypomorphic mutations that impair but do not abolish the function of NEMO are associated with several clinical phenotypes in boys that include immunodeficiency and anhydrotic ectodermal dysplasia (EDA), whereas osteopetrosis and lymphedema are present in a subgroup of patients (classified as X-linked anhidrotic ectodermal dysplasia with immunodeficiency [XL-EDA-ID] or XL-O-EDA-ID if osteopetrosis is present).

XL-EDA-ID patients are susceptible to infections caused by a wide range of organisms that include pyogenic bacteria, atypical mycobacteria, other intracellular organisms, and viruses (e.g. CMV, HSV). The infections manifest themselves as sepsis, pneumonia, sinusitis, otitis, lymphadenitis, stomatitis, pharyngitis as well as (viral) meningitis and encephalitis. Immunologic evaluation reveals impaired specific antibody production, hypogammaglobulinemia with elevated IgM and reduced memory B-cells (akin hyper IgM syndrome), varying degrees of T-cell lymphopenia, abnormal T-cell proliferation, and abnormal cytolytic function. Immunodeficiency results from impaired NF-kB activation upon stimulation by various receptor systems, such as belonging to the Toll-like receptor (TLR), interleukin-1 receptor, and tumor necrosis factor (TNF) receptor families, as well as by T- and B-cell receptors.

Treatment of NEMO: see side box

The observations made in this patient have led to subsequent evaluations in three other patients with defects in NEMO. They showed a similar pattern; reduced expression (percentage and MCF) of perforin (with normal perforin gene) and reduced memory B cells, detected by immunophenotyping. Their clinical presentations varied from selective polysaccharide antibody deficiency to recurrent mycobacterial and pseudomonal infections. These two, straightforward, flow cytometic assays could provide a useful screening tool for NEMO defects: reduced perforin expression plus reduced memory B-cells in boys invites consideration of XL-EDA-ID. This case can be summarized as: "NEMO; immunophenotypically found by coincidence".

Cincinnati Children's

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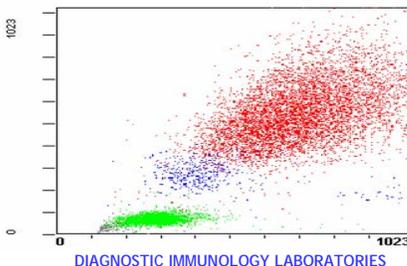Julie Beach
Cincinnati Children's Hospital Medical Center
Hematology/Oncology CHRF1301
3333 Burnet Avenue, Cincinnati, OH 45229
Please call 513-636-4685 with tracking # prior to shipping

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 fax or send results of a same day CBC/diff when ordering these tests*
Shading indicates those tests that MUST also have a Lymphocyte subset analysis ordered

Test	CPT	Price	Sample
❑ *ALPS panel	CPT #88185x9	$403	3ml (1ml) EDTA
❑ Antigen Stimulation	CPT #86353	$444	10ml (5ml) Sodium Heparin
❑ CD40L (CD154)	CPT #88184, 88185x3	$156	5ml (3ml) Sodium Heparin
❑ CD45RA / CD45RO	CPT #88184, 88185x4	$389	3ml (1ml) EDTA
❑ CD59 (PNH Screen)	CPT #88184	$85	3ml (1ml) EDTA *ON ICE*
❑ CD64 (Leuko64)	CPT #88184	$48	1ml (0.5ml) EDTA
❑ CD132 / CD127	CPT #88184, 88185x5	$468	3ml (1ml) EDTA
❑ CTL function	CPT #86849	$471	10ml (5ml) Sodium Heparin
❑ Cytokines, Intracellular (IL-4, IFN-γ, TNF-α)	CPT #88184, 88185x2	$910	3ml (2ml) Sodium Heparin
Note: due to the nature of this assay, specimens must be shipped using First Overnight service so that they are received by the lab by 9:00AM.			
❑ Cytokines, Plasma (panel of 10)	CPT#83520	$1387	5ml (3ml) EDTA
❑ EBV immortalized cell line	CPT #86849	$172	3ml Sodium Heparin
❑ Check here if this is for research purposes only; signed consent must accompany sample			
❑ Lymphocyte Activation Markers	CPT #88184, 88185x8	$559	5ml (2ml) Sodium Heparin
❑ *Lymphocyte subset analysis (CD3,4,8,16/56,19) call for CPT codes		$302	3ml (1ml) EDTA
❑ Mitogen Stimulation	CPT #86353	$667	10ml (5ml) Sodium Heparin
❑ Neutrophil function tests (specify from the list below) **must be scheduled in advance** **not available to non-local customers			
❑ Oxidative Burst ❑ Adhesion Markers ❑ **Phagocytosis & Killing ❑ **Chemotaxis			
❑ NK function	CPT #86849	$377	10ml (5ml) Sodium Heparin
❑ Perforin/Granzyme B	CPT #88184	$406	3ml (1ml) EDTA
❑ SAP protein (for R/O XLP)	CPT #88184	$411	3ml (1ml) Sodium Heparin
❑ Soluble CD163			
❑ Soluble IL-2Rα	CPT #83520	$209	3ml (1ml) EDTA or Na Hep
❑ TCR αβ / TCR γδ	CPT #88184, 88185x4	$453	3ml (1ml) EDTA
❑ TCR V beta clonogram	CPT #88185, 88185x23	$728	3ml (2ml) EDTA
❑ *Wiskott-Aldrich Syndrome Protein (WASP)	CPT #88184	$190	5ml (3ml) Sodium Heparin
❑ ZAP70 for SCID	CPT # 88184	$218	3ml EDTA or NaHep
❑ Other _____			

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