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.

A Confusing Case of Class Switching?

Two brothers were seen in our Immunodeficiency Clinic for an evaluation of possible X-linked Hyper-IgM (XHIGM; a.k.a. HIGM1) syndrome (see side-bar on page 8 for HIGM classification).

The oldest brother (12 at the time of evaluation) was suspected of having HIGM syndrome based on his clinical phenotype - recurrent sinus and ear infections - and the presence of high IgM levels and reduced to absent IgG, IgA, and IgE. A previously performed CD40L assay (a screening assay for HIGM1) was reportedly abnormal. The younger brother (2 yrs old) was diagnosed shortly after birth, based on a similar pattern of increased IgM reduced IgG, IgA and IgE. Both patients receive IVIG and bactrim for PCP prophylaxis.

While the CD40L assay was in progress, we reviewed their B-cell panels. The figures, shown below, represent the percentage of CD27+ B cells (on gated B-cells). In the older brother, 14% of B cells expressed CD27, while the younger brother showed a percentage of 12% CD27+ B cells.

As discussed in our Spring-2006 Newsletter, CD27+ B cells are measured as part of the comprehensive B-cell panel, to determine the percentage of memory B cells in peripheral blood. As shown in the figure on page 7, the percentage of memory B cells in peripheral blood is age-dependent, and typically constitutes >10% of B cells. It can be as high as 25-35% of B cells, even at a young age (and independent of prior vaccinations).

CD27-positive B cells in Hyper IgM Syndrome

12-years Old

Gated on B cells

CD27 FITC

2-years Old

Gated on B cells

See Part 2— Discussion on pages 7 and 8
From the Diagnostic Immunology Laboratories

Clinical Immunology Laboratory | Research Immunology Laboratory

New Assay Development

Phospho-STAT5 (pSTAT5) ASSAY

Introduction:
Protein phosphorylation is an important pathway for regulation of protein function in living cells. It is relevant for the process of signal transduction that regulates gene expression, cellular proliferation and differentiation. Cells respond to the environment in a stepwise mechanism. Upon engagement of a receptor (e.g., growth-, hormone-, cytokine-receptor), molecules are recruited to the receptor to relay the signal to the interior of the cell, resulting in a series of events in response to the outside stimulus. The predominant mechanism by which signal transduction occurs is through tyrosine phosphorylation of intracellular proteins, which in turn induces conformational changes in the proteins, allowing other proteins and molecules to dock and interact with the phosphorylated protein. In the case of signal transduction pathways, phosphorylation is often a transient, reversible, process, indicative of the (sequential) activation of proteins within a cascade of transduction events.

Thus, measurements of phosphorylation of specific intracellular (intranuclear) proteins provides valuable information regarding the specific pathways that have been engaged, including the origin of the cellular activation as well as the kinetics of the response to the outside stimulus. It has been well recognized that defective phosphorylation is linked to a variety of pathological states, including cancer, asthma, diabetes, inflammatory and immunodeficiency disorders.

Consequently, recognition of this central importance of protein kinase dysfunction has spurred development of specific kinase inhibitors to target certain diseases (e.g., imatinib mesylate (Gleevec) as an inhibitor of the Bcr-Abl signaling protein in chronic myeloid leukemia). Thus comparison of differences in phosphorylation events between healthy and diseased cells could be used to identify aberrant intracellular behavior that underlies certain disorders, both from standpoint of diagnostics and therapeutics.

Thanks to advances in the field of flow cytometry (FCM), multi-parameter phospho-FCM is now available to characterize multiple phosphorylated molecules simultaneously at the single cell level, using cells from all relevant tissues (see references 1-4; side-bar). An increasing number of phospho-specific antibodies recognizing phospho-epitopes (e.g. phospho-tyrosine and -serine) are now available, as are their non-phosphorylated counterparts. These include proteins belonging to the mitogen-activated protein (MAP) cascades, the Janus kinase/Signal transducer and activator of transcription (Jak/Stat) cascades, and tyrosine kinase receptors. In addition to new FCM reagents, other innovative developments include improved methods for permeabilization, allowing the reagents to bind to the phosphorylated proteins, new approaches to visualization and analysis of data (akin those used in proteomics), and robotics platforms to facilitate high-throughput acquisition of data.
Jak/Stat Pathway:
The Jak/Stat pathways are especially relevant in the study of immunodeficiency and inflammatory disorders. A multitude of cytokines and growth factors converge in an hour-glass fashion, using limited combinations of Jaks and Stats to transduce the signals down-stream and elicit a multitude of immune/inflammatory responses. Simplified, binding of the cytokine/growth factor to the receptor triggers activation of a specific Jak, which subsequently phosphorylates tyrosine residues on the receptor (see figure on the previous page). This creates sites for interaction with matching Stat proteins that are recruited to the receptors. In turn, the Stats are tyrosine-phosphorylated by the Jaks. These phosphotyrosines then act as docking sites for other Stats, mediating their dimerization into hetero- and homo-dimers. The dimers accumulate in the cell nucleus and activate transcription of their target genes (in an hour-glass fashion).

Detection of Stat phosphorylation by phospho-FCM can test the integrity of the Jak/Stat-dependent pathways in a variety of inflammatory and immunodeficiency disorders. Some of these disorders lend themselves for relatively straightforward screening by phospho-FCM of relevant members of the Jak/Stat cascades (see reference 5). Phospho-FCM can test the transduction pathway used by the common gamma chain family of cytokines in lymphocytes. Cell surface cytokine receptors of this family include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-12, while Jak3 and Stat5 represent the convergence of the signal transduction pathway.

The lethal immunodeficiency disorder; X-linked severe combined immunodeficiency (X-SCID) is caused by mutations in the gene (located on the X chromosome) that encodes the common gamma chain (GC) subunit shared by these cytokine receptors. Boys who have a mutation in this gene, lack T cells and NK cells and have defective B-cell function (despite the presence of B cells). These defects are linked to defective function of multiple cytokine receptors. In addition to X-SCID, a similar autosomal recessive form of SCID has been identified by linking the GC cytokine receptor family to abnormal cellular responses, mediated by Jak3.

Since both XSCID and Jak3-mutant SCID converge at the level of Stat5, phospho-FCM of Stat5 would provide a screening tool for these conditions. This principle is represented by the phospho-Stat5 (pSTAT5) assay. In this assay, whole blood is transiently stimulated by IL-2 to activate the pathway. This is followed by detection of pStat5 in selected lymphocyte populations by a phospho-specific antibody. In the figure below, an example is shown in a healthy adult. The red line represents pSTAT5 expression in the unstimulated condition; the black line represent pSTAT5 expression in CD4+ T cells following stimulation (60% positive). In addition to screening for SCID, the pSTAT assay can also be used to test in vitro B-cell function, as well as measure (constitutive) stat5 phosphorylation in other cells and conditions, such as mast cells, and malignancies. More about this assay and other phospho-FCM applications in future Newsletters.
AN EXERCISE IN PATTERN RECOGNITION

The immune system depends on pattern recognition in order to detect danger signals and counteract infections. The diagnostic process of immunodeficiency disorders is largely based on pattern recognition as well. This can be relatively straightforward, such as observing absence of B cells in X-linked Agammaglobulinemia (XLA), but can also be considerably more challenging. Part of the design and execution of immunological assays at the DIL is focused on facilitating pattern recognition, and this includes looking at qualitative aspects of data, as well as evaluating data that is not part of the primary intention of the assay (i.e. the data that is not reported).

At regular intervals, we will use the Newsletter to exercise our brains in pattern recognition. Have you discovered an interesting pattern? Share it with us and have it included in a future Newsletter.

1. Skewed CD4:CD8 ratio. An ALPS panel was obtained in a patient with thrombocytopenia. The figure represents a dotplot of CD4+ T cells (right upper quadrant) versus non-CD4+ T cells (left upper quadrant), which in this case corresponds to CD8+ T cells. The CD4:CD8 ratio is about 16:1. The patient was suspected of having Wiskott-Aldrich syndrome (WAS); the WASP (WAS Protein) assay performed at the same time confirmed this diagnosis. The pattern of skewed CD4:CD8 ratios (albeit not always as extreme as in this case), associated with CD8+ T-cell lymphopenia is typical for WAS. Our only recently diagnosed WAS patient, from a series of about 10 patients, who did not reveal this pattern, was an infant with acute CMV infection (also highlighting the ever-present exceptions to a rule and the need to regard data in the context of clinical info).

2. CD21-negative B cells. Sometimes the observed patterns suggest who ordered a particular assay. The predominant population of CD21-negative B cells (figure on next page) is a relatively common pattern, observed in patients with Systemic Lupus Erythematosus (SLE). This is often accompanied with other interesting patterns, including reduced CD5+ B cells, B cells with bright expression of CD27 and the presence of plasma-blasts, defined on the basis of dim CD19 expression in combination with bright expression of CD38 and/or CD138. CD21-negative B cells are also observed as part of B-cell reconstitution following B-cell depletion by rituximab, but in that scenario, the accompanying pattern includes increased CD10+/CD5+ and CD27-negative B cells and no plasma-blasts (see also Spring-2006 and Spring-2007 Newsletters).
3. Bright CD3 expression. As is demonstrated by the top left dotplot, paying attention to qualitative aspects can be revealing. It shows a proportion of T cells expressing a higher level of CD3 fluorescence intensity (A). These cells express the gamma/delta T-cell receptor (TcR), indirectly determined by the lack of expression of the alpha/beta TcR. As discussed in the Fall-2007 Newsletter, gamma/delta T cells often lack CD4 and CD8 (B), and may show increased or decreased expression of other markers, such as HLA-DR and CD27 (C).
Online Tool:
1. Go to www.genetests.org/
2. Click on the Decisions Lab.
3. Search by Gene
   - ELA2
   - WAS

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**News From the Genetics Lab**

**New Diagnostic Tests for Congenital Neutropenia: ELA2 and HAX1**

Two new molecular diagnostic tests will join the menu of the Diagnostic Center for Heritable Immunodeficiencies (DCHI).

The differential diagnosis of neutropenia is extensive and includes both acquired and inherited diseases. Recent progress in unraveling the underlying genetic defects has resulted in the identification of several genetic mutations that are associated with congenital neutropenia. Congenital forms of neutropenia can be roughly classified into isolated severe congenital neutropenia (SCN) and neutropenia as part of congenital disorders (see reference 1; side-bar).

SCN is characterized by peripheral blood neutrophil counts that are consistently below 500/µL, and the so-called maturation arrest in the bone marrow, in which neutrophil differentiation is halted at the promyelocyte/myelocyte stage. The clinical manifestations include omphalitis after birth, recurrent skin and oropharyngeal infections, as well as deep-seated abscesses in liver, lung and subcutaneous tissues, amongst other infections. Individuals with SCN have poor wound healing and have an increased risk of myelodysplastic syndrome (MDS), associated with monosomy 7, and acute myeloid leukemia (AML). Treatment options include prompt and aggressive treatment of infections, injections with granulocyte colony-stimulating factor (G-CSF), periodic bone marrow studies to detect MDS, and allogeneic stem cell transplantation (aSCT).

A review of the literature (summarized in reference 2) suggests that 38-80% of autosomal dominant cases of SCN are linked to heterozygous ELA2 mutations. ELA2 encodes neutrophil elastase, a serine protease exclusively expressed in neutrophils and monocytes. How ELA2 mutations cause SCN remains unclear. In addition to SCN, ELA2 mutations also cause cyclic neutropenia; defined as neutropenia with counts below 200/µL for 3-5 days at ~3-week intervals, while peak neutrophil counts often remain below 2000/µL.

The ELA2 mutation detection rate for cyclic neutropenia is higher than for SCN (~90-100% of cases). Genotype-phenotype relationships appear to indicate that certain mutations are predominantly associated with cyclic neutropenia, with no established risk of evolution into AML, whereas other mutations are more commonly found in SCN. It should be realized that the patterns of mutations in cyclic neutropenia and SCN are distinct when populations are studied, but can overlap when individual patients are evaluated. This suggests that distinguishing between cyclic neutropenia and SCN should be done on the basis of clinical findings and not on the basis of genotype alone.

Approximately 50 years after the first description of Kostmann syndrome, mutations in the gene encoding HAX1 were found in the original family. Homozygous mutations in HAX1 are associated (non-overlapping with ELA2) with autosomal recessive cases of SCN. HAX1 encodes a protein critical for maintaining and stabilizing the inner mitochondrial membrane potential and protecting myeloid (progenitor) cells from undergoing apoptosis. As also observed in ELA2 mutations, patients with HAX1 have been shown to acquire somatic mutations in the G-CSF receptor-3 gene (GCSF3R), linked to the process of malignant transformation (reference 4). Genotype-phenotype relationships for HAX1 mutations are becoming apparent. Most patients identified to date are of Middle-Eastern descent, and the vast majority carry a specific mutation (p.Trp44X). Several new mutations have recently been described, associated with the presence of an alternative splice variant (isoform-b) of HAX1, which is also affected by the mutations.

Other neutropenia-associated genes include WASP (part of the DCHI menu), while GF11 (and others) will join the DIL Menu in the near future.

For more information on our DCHI, visit us at ASPHO and FOCIS, or visit us online at: http://www.cincinnatichildrens.org/dchi
The presence of CD27+ B cells in both patients was unexpected, since HIGM1 patients typically lack memory B cells in peripheral blood (compare with figures below, obtained from a 8 year old HIGM1 patient, and four controls with increasing age [CD27 on Y-axis]). The results of the CD40L assay provided the context for these results, as both patients showed normal baseline CD40L expression, as well as normal upregulation of CD40L expression following T-cell activation. The CD40L gene was sequenced in both patients to verify the discrepancy between these and previously obtained results. No mutations in the gene encoding CD40L were identified.

In light of the clinical phenotype and immunological phenotype - our workup showed normal to slightly increased IgM levels and near-absent IgA and IgE levels, other forms of HIGM were considered. Given the apparent X-linked inheritance pattern, mutations in the gene encoding NEMO were ruled out, while normal CD40 expression (also part of the B-cell panel) ruled out HIGM3. Subsequent genetic testing of the gene encoding Activation-Induced Cytidine Deaminase (AICDA) revealed bi-allelic mutations, consistent with a diagnosis of HIGM2, while the gene encoding uracil-N-glycosylase (UNG, HIGM5) was normal.

Hyper-IgM syndromes are due to so-called B-cell intrinsic immunoglobulin class switch recombination (Ig-CSR) deficiencies (see reference 1 in side-bar). CSR is important during the germinal center reaction to improve the response to infections, through isotype-switching from IgM to IgG, IgA, or IgE (with specialized functions, e.g. complement activation or presence in mucosal secretions). The germinal center reaction also includes somatic hypermutation (SHM), which incorporates mutations in the immunoglobulin molecule in a stochastic high-frequency manner, and increases the affinity of the molecule for its antigens.

Both CSR and SHM occur in the germinal center, but are not dependent on each other. Although it has been considered that both CSR and SHM require CD40L interaction on activated T cells with CD40 on B cells, it has been shown that CD40L-independent pathways exist. In the case of CSR, the BAFF system (see Fall-2007 Newsletter) can substitute for CD40L/CD40 (with appropriate cytokines and B-cell antigen receptor engagement), while SHM has been found in IgM-expressing B cells, residing in the splenic marginal zone (representing a defense mechanism against blood-borne encapsulated bacteria).
From the Diagnostic Immunology Laboratories

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From the Diagnostic Immunology Laboratories

Part 2—Continued

Going back to the issue of CD27+ B cells in HIGM syndromes; it has been proposed that peripheral blood memory B cells are derived from B cells that have (successfully) undergone SHM during the germinal center reaction. Immunophenotypically, they express CD27. While in many cases, the lack of CD27+ B cells is indicative of a defective generation of memory B cells (see figure on page 4 of the Spring-2006 Newsletter), the reverse - presence of CD27+ B cells indicative of the presence of memory B cells - is not the case, as demonstrated by HIGM2 patients. In the case of Ig-CSR deficiency on the basis of autosomal recessive AICDA defects, many patients show a normal percentage of CD27+ B cells; yet these cells lack acquisition of somatic hypermutations.

The situation, however, is more complex as AICDA mutations located in the C-terminal part of AICDA have been found in which only CSR is defective, but SHM is preserved. In these cases, the presence of CD27+ B cells may thus appropriately reflect the presence of memory B cells. Lastly, autosomal dominant transmission of AICDA mutations have been found in several patients with variable immunodeficiency phenotypes, and preserved SHM in some, but not all, patients. The mutations affect the nuclear export signal (NES) domain of AICDA. CD27+ B cells are present in these patients.

An important clinical clue in the evaluation of HIGM patients is the presence of lymphoid hyperplasia in certain patients with HIGM2. Pathological examination of biopsied lymph nodes revealed the presence of giant germinal centers, filled with highly proliferating B cells (also referred to as progressive transformation of germinal centers). This histopathological entity is characteristically found in ALPS and in forms of Hodgkin’s lymphoma. Of note, the older brother had shown lymphadenopathy, as well as tonsillar hypertrophy in the past.

Obtaining an accurate (genetic) diagnosis has practical and prognostic implications. These include the association with autoimmunity, and liver disease (associated with Cryptosporidium infection), the risk of PCP pneumonia (and need for PCP prophylaxis), and the risk of lymphoma that are relatively well characterized in HIGM1, but - so far - do not appear to be present in HIGM2. On the other hand, HIGM2 (as well as the other HIGM forms) are somewhat “new” disorders. And thus, more needs to be learned about these, and other conditions of defective CSR and/or SHM. From a practical standpoint, CD27 measurement on B cells should be regarded with caution, and in the context of other information. Other (flow-based) methods are needed as well in order to measure memory B cells (see future Newsletter).
RECAP

In this Newsletter, the following assays were discussed:

1] B cell Panel
Flow cytometry-based panel to provide an immunophenotypic overview of B-cell development and differentiation, representing the main circulating B-cell subsets. Also used to monitor B-cell reconstitution following B-cell depletion. See also Spring-2006 Newsletter and Spring-2007 Newsletter.

2] CD40L Assay
Flow cytometry-based screen for HIGM1, measuring expressing and up-regulation of CD40L on activated T cells. Assay includes measuring expression/up-regulation of ICOS to screen for ICOS defects, as well as to determine overall integrity of T-cell activation. See also Patient Vignette in Fall-2007 Newsletter and Spring-2006 Newsletter (ICOS).

3] pSTAT5 Assay
Flow cytometry-based assay to measure phosphorylation of Stat5 in vitro. It is intended to complement current screening assays for SCID. It will officially be added to the DIL Menu in the near future, but is currently available by request (contact the DIL for details).

4] ALPS panel
Assay to screen for ALPS and determine nature of DNTC populations. See also Fall-2005 Newsletter and Fall-2007 Newsletter.

5] WASP Assay

6] sCD163
ELISA-based assay to measure soluble CD163; a biomarker for activated macrophages and histiocytes in hemophagocytic disorders, such as HLH and macrophage activation syndrome (MAS). sCD163 correlates inversely with cell surface CD163 expression on monocytes that can be measured with the (flow-based) Leuko64 assay.
FEEDBACK

We would like to hear from our Customers. We invite you to share your questions and comments with us. This can be regarding existing assays, new assays that you might be interested in, the way we report results, other services that we can provide, etc. Feel free to send/fax/email your comments to us (fax: 513-636-3546; email: immunodeficiencies@cchmc.org).

CONVENIENT ACCESS TO PATIENTS’ RESULTS: LABTEST.COM

In the Spring-2007 Newsletter, we introduced LABTEST.COM

This Web-based program provides a platform for easy and fast access to patients’ results. Using any standard internet browser, Labtest.com enables you to view reports from the Diagnostic Immunology Laboratories the moment they are generated. There’s no need to wait for a faxed report. For more information or to establish an account, contact our customer service representative; Julie Beach, at 513-636-4685 or julie.beach@cchmc.org

THE SOLUBLE CD163 ASSAY IS BACK!

Back on the DIL Menu: the soluble CD163 (sCD163) assay

CD163 is a member of the cysteine-rich scavenger receptor superfamily. It is a protein that is almost exclusively expressed on human monocytes and macrophages and their derivatives. It is cleaved by a matrix metalloproteinase into a soluble form in response to pro-inflammatory stimuli. It has been determined that increased levels of soluble CD163 (sCD163) are specific for macrophage activation in the context of HLH, and not reflective of inflammation per se. In combination with soluble IL-2Ralpha levels, measurement of sCD163 is helpful in determining histiocyte activation (in parallel with measuring ferritin) in HLH and macrophage activation syndrome (MAS); at baseline and in response to HLH therapy (see also Blessing et al. Arthritis Rheum. 2007;56:965).

The sCD163 assay has been completely overhauled, using new reagents. In addition, a new age-appropriate reference range has been established. In cases of low sCD163 levels, but suspected HLH/MAS activity, cell surface expression of CD163 on monocytes can be determined, using our CD64 assay (Leuko64 kit, see Fall-2005 Newsletter) as a control measurement, given the inverse relationship between cell surface and soluble CD163.

COME VISIT US AT ASPHO AND FOCIS!

The DIL is a component of the Diagnostic Center for Heritable Immunodeficiencies (DCHI; http://www.cincinnatichildrens.org/dchi). The DCHI will be present at the 21st Annual Meeting of the American Society of Pediatric Hematology/Oncology (ASPHO), May 14 — May 17, in Cincinnati, as well as at the Annual Meeting of the Federation of Clinical Immunology Societies (FOCIS), June 5 — June 9, in Boston.
CURRENT MENU OF AVAILABLE TESTS

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*Note: Samples must be received within 24 hours of being drawn. Send at room temperature unless otherwise noted and send using FIRST OVERNIGHT PRIORITY SHIPPING.

Julie Bentley
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*We are now requesting that samples be sent using the FIRST OVERNIGHT option to ensure timely delivery.

Informative newsletters are available at www.cchmc.org/immunodeficiencies