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 Case of Mistaken Immunologic Identity?

In the evaluation of suspected primary immunodeficiency disorders, screening assays are particularly helpful to guide the diagnostic workup. This includes developing strategies for targeted (and cost-effective) genetic testing. In addition, these assays add relevance and significance to abnormal genetic analyses by linking the genotype of a particular genetic sequence variation (or variations) to the clinical and immunological phenotype. Examples of screening assays performed in our Laboratories include measurement of intracellular perforin and granzyme B for hemophagocytic disorders, detection of the gamma chain of the IL-2 receptor (CD132) on lymphocytes for X-linked SCID, amongst many others.

The figures on this page depict results of our CD40L (a.k.a. CD154) assay. This assay screens for the presence of the X-linked form of Hyper IgM syndrome (HIGM1). The basic immunological defect in HIGM1 concerns defective CD40L up-regulation on activated (CD4-expressing) T cells. The top two histograms represent two patient samples, while the bottom histogram shows a healthy control for comparison. The green lines correspond to CD40L expression on (CD4+) T cells at baseline, while the blue lines show up-regulation of CD40L after 5 hrs of polyclonal T-cell activation (numbers denote the percentage of gated T cells that express CD40L). The assay also measures up-regulation of ICOS (see second Newsletter; Spring, 2006) and CD69 to ensure that lack of CD40L up-regulation is not the consequence of more global T-cell dysfunction.

See Part 2 — Discussion on page 7
Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal hematopoietic stem cell disorder. The basic defect in PNH is an expansion of hematopoietic stem cells carrying a mutation in the phosphatidylinositol glycan class A (PIG-A) gene. Because of this mutation, certain types of proteins are unable to be expressed on the surface of the cell due to defective assembly of the glycosyl-phosphatidylinositol (GPI) anchor, a necessary step in surface attachment of certain proteins. Proteins affected by this defect include CD55 and CD59 (regulators of complement activation), CD52, CD14, and others.

The clinical picture of PNH is characterized by a triad of - varying degrees of - intravascular hemolysis, thrombosis and bone marrow failure. Red blood cells lacking (partially or completely) GPI-anchored CD55 and CD59 are unable to regulate and terminate complement activation, thereby making the cells susceptible to lysis by the terminal complement complex. This causes intravascular hemolysis, the primary clinical manifestation in PNH. Hemolysis is chronic with periodic exacerbations (paroxysms), which can lead to the need for frequent blood transfusions. The main signs and symptoms of PNH are: fatigue, hemoglobinuria, abdominal pain, dysphagia, dyspnea, jaundice, thrombosis and development of pulmonary hypertension. Bone marrow failure leads to – combinations of – anemia, neutropenia and thrombocytopenia. The clinical course of PNH varies from patient to patient. Some patients are affected only by mild symptoms while others display a more severe clinical phenotype.

Treatment for PNH remains largely supportive with supplements to aid in the regeneration of erythrocytes (e.g. folate and iron). Recently, a humanized anti-C5a monoclonal antibody has shown promise in the treatment of hemolysis. The reason that patients with PNH develop bone marrow failure is not completely understood. It has been proposed that PNH constitutes a permissive state, in which GPI-defective clones may have a survival advantage over normal stem cells that are targeted by an autoimmun destructive process. Alternatively, (abnormal) GPI-negative stem cells in the bone marrow may pose a target for immune recognition and subsequent destruction. Bone marrow failure manifestations can be treated by similar immunosuppressive therapy as used in other scenarios of bone marrow failure.

Published guidelines recommend that patients with aplastic anemia, refractory anemia-myelodysplastic syndromes, and patients with any one of the following - venous thrombosis involving unusual sites, hemoglobin in the urine, intravascular hemolysis without antibodies, or episodic abdominal pain, dysphagia and hemolysis - should be screened for PNH. Diagnosis of PNH in the “pre-Flow Cytometry” era was accomplished with the Ham’s test and sucrose lysis assay. However, as has become clear with the development of flow-based methods, the latter assays were sensitive enough to detect subclinical PNH, or small numbers of GPI-deficient cells.

Currently, the diagnosis of PNH, as performed in the Diagnostic Immunologic Laboratories, depends on the flow cytometric detection of cell surface expression of molecules that are dependent on the GPI structure, such as CD52, CD55 and CD59 on leukocytes and erythrocytes. This method is particularly useful in the quantification of abnormal cells (“PNH clones”). Thus, it is possible to follow progression or regression of the GPI-negative clones amongst the different cell populations, in the context of the clinical phenotype and independent of recent blood transfusions (if leukocytes are analyzed).
Moreover, it has been determined that GPI-negative cell populations can often be detected in patients with bone marrow failure syndromes, who lack the typical clinical manifestations of PNH. Lastly, the presence of these clones in patients with myelodysplastic syndromes, complicating bone marrow failure, has been linked to a more favorable response to immunosuppressive therapy.

Our Laboratory has recently adapted the PNH assay to include a reagent that directly identifies the GPI anchor, independent of the cell surface structure it is attached to. Aerolysin is a toxin derived from the Aeromonas hydrophila bacterium. Proteolytic cleavage by cellular proteases converts inactive pro-aerolysin into active aerolysin. The introduction of 2 mutations that disrupts the lytic potential, but retains its GPI-binding capacity, together with the coupling to a fluorescent marker created the FLAER reagent. FLAER is highly sensitive and specific in the detection of GPI-deficient cells, regardless of the cell surface receptor it is part of. Due to a lack of proteases, FLAER is not a relevant marker for erythrocytes.

As can be seen in the figure on this page, the fluorescence intensity difference between positive and negative cells is considerably greater with FLAER than with anti-CD59. Moreover, there are conditions in which there is down-regulation (loss) or lack of CD59 (and CD55) expression, unrelated to PNH. An example is illustrated in the figure, showing a lymphocyte population with reduced CD59 expression, but no difference in FLAER staining. This pattern has been observed in a variety of patients with immune activation, predominantly, but not exclusively, affecting lymphocytes. Further exploration in this patient (with DiGeorge syndrome and autoimmune cytopenias) has revealed that the CD59-negative fraction constitutes a T-cell population.

Thus, a combination of FLAER and surface expression of CD59 (and other molecules) provides a reliable method to diagnose PNH, to enumerate the size of the PNH clones (as small as <1% GPI-negative cells) and to distinguish between CD59-negative (or CD52, after treatment with anti-CD52 [e.g. campath] monoclonal antibodies) and GPI-negative cell populations as part of PNH.
Over the past several years there has been a remarkable increase in the number of patients being diagnosed with a variety of eosinophilic diseases. Here at CCHMC, we have launched the Cincinnati Center for Eosinophilic Disorders (CCED; see www.cchmc.org/cced). The mission of the CCED is to provide state-of-the-art patient care linked to innovative bench and bedside research. As such, studies are being conducted to identify non-invasive biomarkers for disease diagnosis and staging, as well as genetic and molecular-immunological tests with diagnostic and therapeutic relevance. Coined as "Eosinophil-based Laboratory Tests" two assays have recently been added to our menu of testing, while others are planned for release in the future (section contributed by Dr. Marc Rothenberg).

In contrast to other parts of the gastrointestinal tract, the normal esophagus does not contain eosinophils, and thus demonstrating eosinophils in this particular anatomical structure is abnormal. Among the disorders to consider (e.g. eosinophilic gastroenteritis, chronic esophagitis, parasitic infection, hypereosinophilic syndromes and gastroesophageal reflux disease [GERD]), eosinophilic esophagitis (EE) is characterized by particularly high eosinophil counts. Current concepts in EE indicate that this is an emerging disorder, likely due to both an increase in disease incidence and an increased recognition of EE as a unique disorder. In children, the incidence is between (at least) 1:1500 and 1:10000, with a peak incidence among Caucasians from industrialized countries. The complex pathogenesis of EE is being unraveled by detailed multidisciplinary clinical studies, genome-wide gene expression profiling, as well as the use of experimental animal models.

Although a proportion of patients with EE are non-atopic, allergic components, demonstrated by food and/or aeroallergen hypersensitivity (extrinsic contribution), as well as a local overproduction of Th2-type cytokines (e.g. IL-4, IL-5, IL-13) in the esophagus (intrinsic contribution), constitute important aspects of the pathogenesis. The gene profiling approach has provided important clues to the pathogenesis, showing a relatively uniform gene transcription profile ("transcriptome") in both atopic and non-atopic EE patients (also conserved across age and sex), as well as a potential role for eotaxin-3, the most highly induced gene in EE tissues.

Clinical manifestations of EE include chest and abdominal pain, dysphagia, heartburn, vomiting, weight loss and food impaction. It is becoming apparent that EE requires long-term therapy and treatment guidelines are currently being developed (see side box). Therapy that modifies disease pathogenesis is highly desirable. In the context of the considerable diagnostic and therapeutic challenges of EE, it is important to have noninvasive tools to complement and/or replace (serial) endoscopic biopsy examinations, in order to diagnose and distinguish EE from other disorders (see above), as well to follow its natural history and monitor response to treatment, especially in the framework of clinical trials using novel therapeutics.

EDN and Eotaxin-3
A series of studies have identified several biomarkers of promise, including (combinations of) peripheral blood absolute eosinophil count (AEC) and plasma levels of eosinophil-derived neurotoxin (EDN) and eotaxin-3. EDN is a major constituent of eosinophil granules and its expression is unique to eosinophils. It is a cytotoxic granule with ribonuclease activity, released in a variety of bodily fluids (serum, urine, stool) in certain inflammatory conditions (e.g. asthma, inflammatory bowel disease). Eotaxin-3 is one of a family of eosinophil-specific chemokines, which as mentioned above, has been identified in tissue-specific gene profiling assays as an important etiologic factor in the pathogenesis of EE. Moreover, the levels of eotaxin-3 transcripts correlate with disease severity, and appear to be linked to a single nucleotide polymorphism (SNP) in the eotaxin-3 gene (to be discussed in a future Newsletter edition).

Both molecules are measured in plasma using ELISA methodology and are compared to age-appropriate healthy control values. In combination with diagnostic endoscopy and other biomarkers, EDN and eotaxin-3 may demonstrate usefulness in the diagnostic process of EE, as well as in monitoring its natural history and response to therapeutic interventions, particularly if these target the pathogenesis of EE.
Immunophenotyping of cells of the immune system is a staple in the workup of patients with immunologic disorders. According to the principle of “Form Follows Function”, immunophenotyping is reflective of functional characteristics of the immune system (or lack thereof). Progress in immunophenotyping continues to be made, as demonstrated by the fact that new lymphocyte subsets, as well as important new characteristics of known subsets are discovered in a seemingly unrelenting pace. Ongoing developments in contemporary flow cytometry have been helpful in changing concepts of “multi” in multi-parameter flow cytometry, paralleled by continued refinement in analysis software, and a continued expansion of the repertoire of commercially available reagents. To illustrate the enduring power of immunophenotyping, examples of innovations will be featured on a regular basis in the Newsletter. Consistent with the mission of the DIL, these innovations will make their way into the menu of available tests.

One of the most significant discoveries in T-cell phenotyping has been the detection of alternatively spliced isoforms of the cell surface receptor CD45. The fact that these isoforms are differentially expressed on specific T-cell subsets is widely used in the phenotypic characterization of T cells, despite the fact their receptors remain largely unknown. The basic premise is that the long isoform of CD45, CD45RA, is expressed on naive T cells, while the shorter isoform, CD45RO, is expressed on T cells that have encountered (i.e. primed by) antigens.

In clinical immunology, this distinction between CD45RA+ versus CD45RO+ T-cell subsets is particular useful to determine the status of the naive T-cell compartment relative to their thymic origin (i.e. the status of the thymus). Primary immunodeficiency disorders characterized by decreased/absent thymic output often show decreased CD45RA+ T cells (see figure a). As can be seen in the upper dotplots, obtained from a healthy control (HC), CD4 and CD8 T cells can be divided into CD45RA+/CD45RO- cells (naïve cells; R3/R7), CD45RA-/CD45RO+ T cells (primed cells; R5/R9) and, typically, a small population of CD45RA+/CD45RO+ T cells (“transitional” cells; R4/R8). In contrast, the lower dotplots, obtained from a patient (Pt) with a combined immunodeficiency disorder (CID) shows the almost complete lack of naive CD4 and CD8 T-cells (R3 and R7, respectively).

The status of the thymus can similarly be followed using these markers in the context of immune reconstitution post stem cell transplantation. Large-scale immune activation in vivo can also induce shifts in the distribution between CD45RA+ and CD45RO+ T-cell subsets (e.g. EBV infection).

![Figure A](image-url)
An expansion of this basic model incorporates the expression of the chemokine receptor CCR7. Naive cells express CD45RA and CCR7, while antigen-experienced/primed T cells can be subdivided into central memory cells (CD45RO+ and CCR7+/bright; “TCM”) and effector memory cells (CD45RO+ and CCR7-/dim; “TEM”). In figure b, CCR7/CD45RO staining are shown from the patient and control in figure a. In this example one can see that the CD45RO-positive cells are largely CCR7 negative, consistent with a TEM phenotype.

It has become clear that the distinction between naive (CD45RA+) T cells and antigen-experienced (CD45RO+) T cells is quite accurate for T cells expressing CD4, but not for T cells expressing CD8. Although an unidirectional differentiation pathway of CD45RA+ into CD45RO+ cells was originally proposed, it has become clear that certain CD8+ T cells that express CD45RA, are not naive. They are referred to as effector memory cells RA+ (TEMRA). In figure c, an example is shown from a patient with an immunodeficiency disorder, affecting T-cell generation and function. The upper left dotplot shows a lack of naive (CD45RA+) CD4+ T cells (R5).

The CD8+ T-cell compartment seems to show a preserved naïve population, based on the expression of CD45RA (R7), which would suggest that the basic defect in this patient affects CD4+ T cells differently than CD8+ T cells. Adding the CCR7 marker shows that the primed CD4+ T cells indeed have a TEM phenotype (lower left dotplot). However, the presumed naïve CD8+ T cells are not naïve, but are in fact TEMRA cells. Thus, this expanded immunophenotyping model more accurately identifies the relevant T-cell subsets, with respect to thymic origin, antigen experience and effector/memory classification.

There are many more layers of T-cell immunophenotyping that can be added to the model, for example by including markers that identify activation states (e.g. CD71, HLA-DR), (loss of) co-stimulatory receptors (CD27, CD28), cytokine receptors (e.g. CD127), markers of cytotoxicity (e.g. CD57, perforin) and homing/trafficking receptors (e.g. CD62L, CXCR5). This may be the subject of a future “Immuno-101” discussion.

As with all immunologic studies, the power of the assays is determined by how it is used and by how the results are interpreted.

Immuno-101; further reading:
HIGM Syndrome Classification:
- HIGM1: defects in CD40L gene
- HIGM2: defects in AICDA gene
- HIGM3: defects in CD40 gene
- HIGM4: unknown

HIGM: further reading:

Patient Vignette (part 2 – Discussion)

The assays show similar patterns: low CD40L expression at baseline and essentially no up-regulation after stimulation (with normal ICOS and CD69 expression), while the healthy control exhibits the typical pattern of CD40L up-regulation on activated T cells.

Despite a similar lack of CD40L up-regulation, only one of the patient samples was obtained from a patient with HIGM1. The other CD40L assay, illustrated in the middle histogram, was obtained in the context of a broad-based immunologic workup in a patient with autoimmune cytopenias. The patient is a previously healthy 14-year-old male, who presented with a transient episode of autoimmune hemolytic anemia, followed by chronic/relapsing immune-mediated thrombocytopenia (ITP), requiring escalating therapeutic interventions (including the use of rituximab). The clinical history was negative for recurrent and/or chronic infections, including opportunistic infections. The patient showed widespread lymphadenopathy, as well as a mild splenomegaly, both predating the occurrence of ITP. The immunological workup revealed normal levels of isotype-switched immunoglobulins (IgG, IgA and IgE) and a low IgM level. Responses to antibody titers could not be measured due to prior infusions of IVIG and rituximab; no B cells were present in peripheral blood. T-cell and NK-cell function were normal. Genetic evaluation of CD40L showed a sequence variation, characterized by a non-conservative substitution of glycine to arginine in amino acid 219 (G219R). A polymorphism at this position has previously been reported (see side box, #5). Genetic evaluation of the patient’s mother showed that she is a carrier of this sequence variation. Given the fact that her CD40L assay showed the characteristic bimodal distribution seen in carriers of HIGM1 would indicate that this sequence variant may not merely be a polymorphism. A second male patient with recalcitrant ITP (requiring splenectomy), showing absent CD40L up-regulation, as well as the CD40L-G219R sequence variant was recently identified. His evaluation, so far, is similarly not consistent with HIGM1.

Despite an abnormal CD40L assay, no evidence of Hyper IgM syndrome: A case of mistaken immunologic identity?
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 use the blank space below and send/fax/email your comments to us (fax: 513-636-3546; email: immunodeficiencies@cchmc.org).
**IN THE NEXT ISSUE**

- Patient Vignette
- Monitoring B-cell depletion therapy
- More on Eotaxin-3
- CD107: turning cytotoxic molecules inside out
- News from the Genetics Lab

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**CURRENT MENU OF AVAILABLE TESTS**

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<td>Check here if this is for research purposes only: signed consent must accompany sample</td>
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**Sample Information**

- 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 Beach
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  Hematology/Oncology, CIN (ER130)
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**Diagnostic Immunology Laboratories**

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