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.

Bright Expression; Dim Understanding

The patient vignettes in the previous Newsletters could give the impression that we solve all our puzzles. This is clearly not the case, and sometimes the best we can do is to provide a scenario of a possible underlying immunologic anomaly.

The patient vignette in this Newsletter deals with a patient with a perplexing profile, in which it has been a challenge to connect the clinical dots to the immunologic dots. This 4-year old patient was referred to our Immunodeficiency Program for natural killer (NK) cell lymphocytosis. The clinical history is complex and includes prematurity, presumed milk protein allergy and, subsequently, widespread gastritis and colitis with failure to thrive. Workup showed atypical lymphocytosis with increased NK-cells, detected at multiple occasions.

Prior to his visit, the highest recorded NK-cell count was 11,848 cells per microliter. The immunological workup at that point was not normal, but not directly pointing towards a defined primary immunodeficiency disorder.

The NK-cell count at this visit was 3,093 cells per microliter. To further characterize his NK-cell compartment, perforin and granzyme expression profiles were obtained. As is shown in the figure below, the patient displayed an unusual expansion of NK-cells that express CD56 at higher levels (so-called CD56bright cells), but express less perforin.

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Perforin Content Relative to CD56 Expression in NK-cells

Figure: Expanded CD56bright NK-cells with reduced perforin expression in patient (right) and healthy control (left).

See Part 2—Discussion on pages 7, 8
New Assay Development

XIAP-XLP2 ASSAY

Introduction:
In 2007, genetic analysis of BIRC4 was added to the list of genetic tests for Primary Immunodeficiency Disorders (PIDs), offered by the Diagnostic Center for Heritable Immunodeficiencies (DCHI), see Spring-2007 Newsletter. Mutations in BIRC4 (Xq25) underlie X-linked lymphoproliferative disease type 2 (XLP2). Consistent with the triangular approach to PIDs at CCHMC, in which clinical phenotypes are regarded in an interactive context with immunological phenotypes and genotypes, an assay has been developed specifically for the diagnosis of XLP2.

XLP2 is a recently discovered X-linked proliferative disorder. Similar to (“classic”) XLP1, which is caused by mutations in the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (SAP), XLP2 can present with life-threatening EBV-associated hemophagocytic lymphohistiocytosis. In addition to EBV-associated disease, it is likely that additional clinical phenotypes of XLP2 can be found that may or may not overlap with XLP1. Indeed, our early experience with XLP2 includes several patients with autoimmune hepatitis, as well as hemophagocytic lymphohistiocytosis without (viral) etiology. Much more work is needed to establish the potential clinical phenotypes of XLP2.

XIAP-XLP2 Assay:
The XIAP-XLP2 assay detects intracellular presence of X-linked Inhibitor of Apoptosis Protein (XIAP), the product encoded by BIRC4. As the name implies, it functions as an inhibitor of apoptosis and is expressed in many tissues and cells of hematopoietic origin.

Similar to the SAP assay (to be renamed as the SAP-XLP1 assay) the XIAP-XLP2 assay serves two main functions: provide a rapid, flow cytometric screening assay for XLP2 that can be used to pursue genetic testing and initiate specific treatments for XLP, and to allow the study of phenotype to (BIRC4) genotype relationships.

The monoclonal antibody (clone 48) used in the assay recognizes an epitope within the region of XIAP spanning amino acids 268-426. As can be seen in the figure on the next page, XIAP is present in most white blood cell populations (normal control on the left; open histograms represent control antibody). In comparison, XIAP was reduced or undetectable in three XLP2 patients. These patients were found to have a multi-exon deletion, and nonsense mutations, resulting in an early stop codon, respectively.

Thus far, the results of the XIAP-XLP2 assay in patients with missense mutations require a more cautious approach. There was mildly reduced XIAP expression in one patient, but normal expression (in EBV-transformed B-cells) in another patient, who received a bone marrow transplant before the diagnosis was made. In these cases, a more detailed measurement of the amount of XIAP expression by quantitative flow cytometry, for example using Quantum™ MESF beads, may be indicated.

In addition to using this assay to screen for XLP2, early results indicate that carriers of BIRC4 mutations (e.g. mothers of patients) may have bimodal distributions of XIAP-positive and XIAP-negative cells. Lastly, measurement of XIAP after bone marrow transplantation can be used to measure chimerism in specific cell populations (without the need to physically separate the populations of interest).

As with other screening assays, even a normal test result may still warrant mutational analysis of BIRC4. It seems reasonable at this moment that males with clinical manifestations indicative of XLP, particularly EBV-associated disease, undergo SAP-XLP1 testing first, followed, if normal, by the XIAP-XLP2 assay and/or genetic testing of BIRC4.

Lastly, there remains a group of still undefined XLP patients (XLP3 and beyond), inviting additional screening assays to be developed (see future Newsletters).
XIAP-XLP2 ASSAY

Figure: The XIAP-XLP2 assay showing intracellular XIAP in lymphocyte subsets, as well as monocytes and granulocytes, in healthy control and three XLP2 patients with BIRC4 mutations.
Healthy EBV carriers have between 1 and 50 EBV-infected B-cells per million cells, in which EBV is kept in Latency Program (LP)-0 or LP-I. EBV-infected B cells that traffic to the lymphoid follicles, acquire the intermediate, LP-II program (also known as the “rescue program”), which regulates B-cell proliferation and survival (into long-lived memory cells). In the absence of effective EBV-specific immunity, EBV enters LP-III (also referred to as the “growth program”). EBV associated malignancies reflect these distinct programs. For example, LP-I is found in association with EBV-positive Burkitt’s lymphoma, EBV-positive Hodgkin’s lymphoma cells express LP-II, and LP-III is found in EBV-lymphoproliferative disorders in immuno-incompetent individuals.

**EBV – Lytic Phase:**
- Production of infectious viral particles
- Death of infected cells
- Viral DNA polymerase used
- Acyclovir sensitive
- Many lytic viral genes expressed (e.g. BZLF1/BRLF1, BHRF1, BCRF1, BALF2/5, BMRF1)

**EBV – Latent Phase:**
- No production of infectious viral particles; B-cell immortalization
- Viral DNA maintained as circular episome
- Cellular DNA polymerase used for low-level replication
- Acyclovir insensitive
- Restricted set of latency genes expressed, according to a latency program (LP)
  - LP-0: (LMP2A)
  - LP-I: LMP2A, (EBNA1)
  - LP-II: EBNA1, LMP2A, LMP2B, LMP1
  - LP-III: EBNA1, LMP2A, LMP2B, LMP1, EBNA2–EBNA6

EBV is associated with a variety of clinical entities that can be separated in those that occur in immuno-competent and immuno-incompetent hosts (see reference 1). Summarized, it can be associated with a relatively unremarkable viral syndrome in infants and young children, a self-limiting lymphoproliferative disorder, infectious mononucleosis (IM) in teenagers and adults, as well as a variety of B-, T- and epithelial-cell malignancies.

In IM, approximately 1% of B-cells are infected and maintained at the LP-III stage, leading to extensive B-cell proliferation. This leads to nonspecific T-cell activation of CD8+ T cells (the “atypical lymphocytes”). Subsequent development of an EBV-specific T-cell response (directed towards epitopes including EBNA3 and BZLF1) eliminates most infected B-cells, followed by entering the latent phase by the remaining, infected, B-cells.
The crucial role of the cytotoxic T-cell response to EBV is demonstrated by the development of EBV-driven lymphoproliferative disorders. Post-transplantation lymphoproliferative disorders (PTLDs) consist of a group of (WHO-classified) EBV-associated B-cell proliferations (see side-bar on the previous page). PTLD can occur following bone marrow transplantation (BMT) or following solid organ transplantation (SOT), often affecting extranodal sites and transplanted organs. The cited incidence of PTLD varies between 1% and 33%. Risk factors for PTLD include EBV seronegativity before transplantation, primary EBV infection post-transplantation, degree of immunosuppression, type of transplant, concomitant presence of cytomegalovirus and younger age.

Longitudinal studies in PTLD have shown a progressive switch from a restricted LP program - as seen in healthy carriers - to broader patterns, including LP-III. Cytotoxic T-cell function is an important parameter for this progression, as withdrawal of immunosuppression and/or therapeutic infusion of EBV-specific T-cells can lead to regression. Progression of initial PTLD lesions into lymphoma signals a phase in which other genetic/epigenetic changes within the host genome are acquired (e.g. affecting c-MYC, or aberrant promoter hypermethylation).

**SET/EBV further reading:**

The characteristic of EBV as the only known agent capable of B-cell immortalization, is commonly used for the in vitro establishment of permanent B-cell lines (LCL). In combination with other measurements of EBV activity, information regarding the occurrence of this immortalization process in vivo could be of benefit in determining the occurrence of PTLD in at-risk patient populations. B-cell transformation can be evaluated by assessing spontaneous EBV transformation (SET) of peripheral blood mononuclear cells (PBMC) in vitro. In renal transplantation patients, the SET assay, showing spontaneous outgrowth of EBV-transformed B cells in vitro, correlated significantly with EBV load and absent EBV-specific T-cells.

The principle of this assay is to determine if PBMCs, taken from patients with suspected EBV-related disorders, undergo spontaneous EBV-transformation in vitro (i.e. whether a LCL can be established without the need to infect the cells with EBV in vitro). EBV is used as a control, to ensure that PBMCs are intrinsically capable to be transformed in vitro. Cells are cultured at a relatively low cell number, predetermined at a level in which spontaneous transformation of PBMCs in healthy seropositive controls is not observed. Phytohemmagglutinin is added to the cultures to exhaust T-cells in this ~2-week culture, thereby excluding the influence of cytotoxic T-cells on the ability of B-cells to be transformed by EBV in vitro.

It is anticipated that the combined application of EBV-DNA load, as determined by real-time PCR (in whole blood), the SET assay and perhaps EBV-specific T-cell enumeration, will provide the tools to better understand and - hopefully - predict PTLD. With increasing knowledge, therapeutic interventions can be instituted before PTLD occurs or progresses into difficult-to-treat lymphomas, while at the same time limiting unnecessary reduction in immunosuppressive drugs that may lead to organ rejection. In addition to PTLD in the context of SOT or BMT, the SET assay can also be used to determine EBV-lymphoproliferative disease in the context of primary immunodeficiency disorders, including X-linked lymphoproliferative disorders, and other T-cell disorders.
The DIL has a long-standing interest in developing collaborative networks with other programs at CCHMC that are engaged in diagnostic services relevant to immunologic and hematologic/oncologic diseases. The **Translational Trial Development and Support Laboratory (TTDSL)** is part of Experimental Hematology and Cancer Biology Translational Core Laboratories at CCHMC. The TTDSL is a CAP/CLIA certified laboratory and provides a wide range of services to both internal and external investigators (see [www.cincinnatichildrens.org/ttdsl](http://www.cincinnatichildrens.org/ttdsl) for detailed information).

**Fanconi Anemia (FA)** is a genetically heterogeneous recessive syndrome characterized by common phenotypic abnormalities that include congenital malformations, progressive bone marrow failure and predisposition to acute myelogenous leukemia and other malignancies. The disease results from defects in genes involved in DNA repair and is associated with chromosomal instability and hypersensitivity to DNA cross-linking agents.

To date, defects in as many as 13 different genes have been identified that result in the FA phenotype (FANCA, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M and -N).

Mutations in the Fanconi Anemia A gene (**FANCA**) account for about 60% of all FA patients. Mutations in the **FANCC** gene and **FANCG** gene represent another 15% and 10%, respectively. The subtypes are also termed FA complementation groups, as complementation of a cell defective in a particular FANC gene by its intact corresponding gene corrects the DNA repair defect.

The diagnosis of FA relies on the demonstration of characteristic clinical phenotypes. Hypersensitivity to chromosomal breakage by the DNA cross-linking agent diepoxybutane (DEB) and/or mitomycin C (MMC) is common to all FA complementation groups, and is the basis for the currently accepted diagnostic test for FA. However, in a significant number of cases, the results of DEB/MMC-chromosomal breakage studies are indeterminate.

To resolve the different FA subtypes, the **TTDSL** has developed the **FA complementation assay**. The FA complementation assay is an efficient method for the identification of specific complementation groups by exploiting the characteristic of FA cells to undergo arrest in the G2/M phase of the cell cycle in response to DNA damaging agents.

**Complementation Analysis in a FA Patient with a FANCA Defect**

![Figure: Complementation by retroviral insertion of intact FANCA gene corrects G2/M phase cell cycle arrest, pointing towards a FANCA subtype in this FA patient](image-url)
Retroviral vector complementation is currently available for FANCA, FANCC, FANCG, FANCE, FANCF, FANCL and FANCB (representing > 90% of the FA patients in the US). Complementation tests for FANCI, FANCM and FANJ are under development.

Briefly, skin fibroblasts or lymphoid cell lines (e.g. LCL) are first complemented with retrovirus mediated transfer of genes for FANC groups -A, -C, and -G, the 3 most common FA subtypes. These cells are exposed for 48 hours to the alkylating agent melphalan, stained with Propidium Iodide (PI) dye and cell cycle analysis for FA-specific G2/M arrest is performed by flow cytometry. All retroviral vectors carrying different FANC genes co-express a fluorescent marker (enhanced green fluorescent protein; eGFP) such that gene-complemented cells are detected by flow cytometry. The percentage of eGFP-positive cells in the G2/M phase of cell cycle for each FANC vector is calculated and compared to the eGFP control vector group. A complementation group is identified when the G2/M arrest is corrected.

The figure on the previous page shows an example of fibroblast complementation in a patient with a FANCA defect. Intact genes encoding FANCA, -C and -G were inserted. Correction occurred by intact FANCA, but not with the FANCC and -G genes, consistent with a genetic defect in FANCA (20% arrested cells versus 55%, 63% and 53%, for the control, FANCC and -G, respectively).

If a DEB-abnormal LCL sample is found not to be sensitive to melphalan, the assay is repeated using a fibroblast cell line that generally remains sensitive to melphalan.

In 85% of cases, a complementation group can be identified in this round of testing. If no complementation occurs with groups A, C and G, additional studies, such as the FANCD2 western blot can be performed.

A key step in the FA pathway is the mono-ubiquitination of the FANCD2 protein, which causes a slightly lower mobility and appears as a second, higher molecular size band on a FANCD2 western blot. Such an analysis allows the identification of defects that are upstream versus those that are downstream of FANCD2 mono-ubiquitination.

In normal cells, mono-ubiquitination of FANCD2 can be induced with hydroxyurea. “Upstream” mutations in FANCA, -B, -C, -E, -F, -G, -I and -L result in deficient mono-ubiquitination and absence of the upper band. In contrast, defects that are “downstream” of FANCD2 mono-ubiquitination (e.g. mutations in FANCD1, -J and -N) result in normal FANCD2 western blot. If the FANCD2 western blot assay indicates an “upstream” defect, we can complement the cells with retrovirus vectors carrying the FANCB, -E, -F, -I and -L genes to identify the specific FA gene defect.

Once a complementation group is identified or the possible groups are narrowed down, sequencing of the respective gene(s) can be done to identify the specific FA-associated mutation.

Information regarding FA complementation testing, as well as DEB testing, can be obtained by contacting the TTDSL at 513-636-0958 or by visiting the website at www.cincinnatichildrens.org/ttdsl.

Patient Vignette (part 2, continued from page 1)

While perforin expression in these CD56bright NK-cells was reduced; granzyme B content was fairly normal. To determine the consequence of this unusual phenotype on function, NK-cell function assays were performed. In our standard (bulk) assay, NK-cells showed robust activity (reported as >100 lytic units). In the second approach, CD107a mobilization was measured.

As described in the Fall-2007 Newsletter, the CD107a assay measures the process of exocytosis of lytic granules (secondary lysosomes), in which CD107a (also known as the lysosome-associated membrane protein-1 [LAMP-1]) fuses with the cell membrane in response to target cells. This assay showed no major differences in CD107a mobilization, relative to CD56 expression level.
Thus, despite the dominating presence of CD56bright NK-cells with reduced perforin expression, there seemed to be no consequences for NK-cell function, which perhaps should not come as a surprise seeing that the number of NK-cells with “normal” CD56 expression and perforin content was within normal limits. The immunologic workup of this particular patient showed subtle abnormalities affecting his T- and B-cell compartments as well, without pointing towards a specific immunodeficiency disorder.

And thus, at this point it remains unclear how NK-cell lymphoproliferation, including expansion of a subset of CD56bright NK-cells that typically constitutes only a minority of NK-cells, and other immunologic abnormalities are connected to the clinical manifestations, dominated by long-standing colitis and failure to thrive.

One can envision several mechanisms accounting for this expansion. For example, it may be a primary consequence of perturbed development and differentiation of NK-cell subsets, or a secondary result of (response to) another pathogenic process, such as local inflammation affecting the GI system.

Two other examples are shown below to illustrate dominant CD56bright NK-cell populations. Following bone marrow transplantation, NK-cell reconstitution is early (before T- and B-cell reconstitution).

The dotplot on the left represents NK-cell status -3 months post-allogeneic transplantation. At that time, NK-cells were expanded and NK-cell function was normal. In the second individual (dotplot on the right), the expanded population of CD56bright NK-cells was found in the context of active colitis and failure to thrive and borderline reduced NK-cell numbers and reduced NK-cell function.

Although it has been proposed that CD56bright NK-cells are (ontogenic) precursors of CD56low NK-cells, a number of recent studies suggest that CD56bright NK-cells have unique characteristics pertaining to cell surface and intracellular phenotypic markers that indicate activation (e.g. higher levels of IL2-Rαβγ, IL7-R), trafficking (e.g. higher levels of CD62L and CCR7; lower levels of CXCR1), and functional status (e.g. absent or lower levels of perforin, granzymes CD16 and KIRs; higher levels of NKG2A). Moreover, CD56bright cells differ in the production of inflammatory mediators (e.g. higher levels of TNF-α, VEGF; lower levels of IL-8 and eotaxin-2), and cytotoxic (lower) versus proliferative (higher) potential.

A recent gene and protein expression study summarized this as: “CD56bright represent an independent cell population that is neither terminally differentiated nor functionally exhausted” (Wendt et, 2006).

CD56bright NK-cells in Immunologic Scenarios

![CD56bright NK-cells in Immunologic Scenarios](image)

*SCID post BMT*, *IBD Patient*  

47% 51%

*Gated on all NK-cells

Figure: Expanded CD56bright NK-cells with reduced perforin expression in SCID patient post BMT (left) and patient with colitis (right).
RECAP

In this Newsletter, the following assays were discussed:

1] **Perforin/Granzyme B Assay**
Flow cytometry-based panel to measure intracellular perforin and granzyme B. This assay is used to screen for genetic (familial) forms of hemophagocytic lymphohistiocytosis (HLH). See also Patient Vignette in Spring-2006 Newsletter and Spring-2007 Newsletter.

2] **CD107a Mobilization Assay**
Flow cytometry-based assay to screen for the presence of familial HLH on the basis of mutations in MUNC13-4, and as a component of NK-cell function studies. See also Fall-2007 Newsletter.

3] **XIAP-XLP2**
Flow cytometry-based assay that measures intracellular XIAP. Assay to screen for XLP2 on the basis of mutations in the gene encoding XIAP (BIRC4). Note: XLP1 (“classic” XLP) can be screened for by the SAP assay (to be renamed into XIAP-SAP assay). See also Patient Vignette in Fall-2005 Newsletter.

4] **SET Assay**
In vitro assay to determine spontaneous transformation of B cells, infected in vivo by EBV. The SET assay is intended to link EBV reactivation to development of post-transplantation lymphoproliferative disorders. It will officially be added to the DIL Menu in the near future, but is currently available by request (contact the DIL for details).

5] **FA Complementation Assay**
Flow cytometry-based assay used to identify genetic subtypes of Fanconi Anemia, based on the principle that complementation of a cell defective in a particular Fanconi Anemia gene (FANC gene) by retroviral transduction with the intact gene corrects the arrest in the G2/M phase of the cell cycle (representative of the DNA repair defect in FA).
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, http://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

ESTABLISHING PEDIATRIC REFERENCE RANGES

The DIL spends considerable resources establishing pediatric reference ranges for its tests. This is not an easy process and we are constantly looking for fresh ideas, new approaches, for example to maintain a diverse representation of healthy pediatric subjects. Got ideas? Share them with us (email: immunodeficiencies@cchmc.org).

GO WEST CAROL MOORE

After 20+ years of dedicated service in the DIL, Carol Moore has accepted an exciting new position as Associate Director of the Flow Cytometry Laboratory at Genoptix in Carlsbad, California. We wish her lots of luck in her new position.
CURRENT MENU OF AVAILABLE TESTS

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