From the Diagnostic Immunology Laboratories

Clinical Immunology Laboratory | Research Immunology Laboratory

Patient Vignette

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 Season of Spring in the DIL

Spring is the season of change; a fresh start; a transition from old into new. The patient vignette in this issue is dedicated to the concept of a fresh start, as experienced at Cincinnati Children’s Hospital Medical Center.

A major emphasis of the DIL is on performing and developing screening assays for primary immunodeficiency disorders. The central theme is that these immunodeficiencies can be — and in many cases should be — corrected by hematopoietic stem cell transplantation (HSCT).

Examples include CD127/CD132, Foxp3, CD40L, WASP, oxidative burst, SAP and perforin/granzyme B (see “Current Menu of Assays” on page 10).

The figures on this page (and continued on page 8) show several examples. Instead of the usual comparison between patients and healthy control subjects, this time the comparison is between before SCT (old) and after SCT (new). For consistency, all figures are shown as single-parameter histograms, with green lines representing either baseline or control antibody staining.

Detection of Engraftment following HSCT by Flow Cytometry

CD132: detects surface expression of the common gamma chain, part of the IL-2 receptor complex. Mutations in the gene encoding CD132 cause the X-linked form of SCID (XSCID).

WASP: detects the intracellular presence of the Wiskott-Aldrich syndrome (WAS) protein, defective in patients with WAS.

Continued on page 8

continued on page 8
B-cell depletion therapy has long been part of the management of autoimmune diseases characterized by autoantibody production. With the development of monoclonal antibody (mAb) therapy, B-cell depletion can be accomplished in a relatively specific manner. Rituximab is a chimeric (mouse/human) monoclonal antibody that targets CD20, exclusively expressed by human B cells. Pioneered for the treatment of B-cell malignancies, its therapeutic repertoire has expanded to include many other scenarios, including EBV-associated lymphoproliferative disease (post bone marrow or solid organ transplantation), rheumatologic disorders, autoimmune cytopenias, solid organ rejection, chronic graft-versus-host disease, vasculitic disorders, among others.

The next generation of B-cell specific monoclonal antibodies, including antibodies directed against CD19 and CD22, are already finding their way into the clinic. Their mechanisms of action, as well those of yet-to-be-developed mAbs may be similar to that of rituximab, but may also be fundamentally different.

These mechanisms determine if and how B cells are eliminated, and, conversely, how B cells, or specific B-cell populations, reconstitute/recover after depletion (and potentially cause relapse). Thus, an important component in the determination whether mAb-mediated B-cell depletion therapy is successful, is evaluating B-cell recovery. The DIL are exploring several approaches that together will form the B-cell reconstitution panel.

When fully implemented, this panel will consist of several components that include (longitudinal) immunophenotyping of B-cell subsets, measurement of constituents of the BAFF system, as well as evaluation of relevant genetic determinants. In this edition, B-cell immunophenotyping will be touched upon, while a more detailed description of the BAFF system, as well as genetic determinants and their roles in B-cell reconstitution, will appear in a future edition. It stands to reason that some or more of these components can be used to monitor B-cell reconstitution following other modes of therapy (e.g. cyclophosphamide) as well.

B-cell Reconstitution Kinetics Vary Widely Between Individuals

Graph showing absolute B-cell counts in 6 individuals treated with rituximab. Subjects are synchronized to pre-rituximab (baseline) levels at time-point 1 followed by subsequent time-points approximately one month apart. Four subjects received rituximab for autoimmune cytopenias; the other two patients were treated for EBV reactivation post transplantation.
The figure on the previous page shows a relatively well-recognized phenomenon: widely variable B-cell recovery kinetics. It has been observed that B-cell counts reach or exceed pre-rituximab levels after only a few months in some individuals, while in others it can take many months. In fact, in several individuals, no substantial recovery of B-cell counts has occurred in more than one year following completion of rituximab treatment.

Two possibilities that may operate in tandem, are considered. The first concerns the **BAFF system**: important for B-cell differentiation, following release of immature B cells from the bone marrow. The second relates to our genetic make-up with regard to **single-nucleotide polymorphisms in the Fcgamma receptor genes** (CD16 and CD32, respectively). Both are considered to become part of the panel (discussed in the next issue of the Newsletter).

**Different B-cell Phenotypes at 3/4 and 5/6 months post rituximab in 2 patients (cells = total B cells)**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Pt1</th>
<th>Pt2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/4-months</td>
<td>49 cells</td>
<td>98 cells</td>
</tr>
<tr>
<td>CD10</td>
<td>4.2%</td>
<td>90%</td>
</tr>
<tr>
<td>CD19</td>
<td>43%</td>
<td>94%</td>
</tr>
<tr>
<td>CD27</td>
<td>18%</td>
<td>2.9%</td>
</tr>
<tr>
<td>5/6-months</td>
<td>175 cells</td>
<td>290 cells</td>
</tr>
<tr>
<td>CD10</td>
<td>14%</td>
<td>64%</td>
</tr>
<tr>
<td>CD5</td>
<td>34%</td>
<td>83%</td>
</tr>
<tr>
<td>CD27</td>
<td>25%</td>
<td>5%</td>
</tr>
</tbody>
</table>
CD5 is included in our panel because CD5 is an age-dependent B-cell marker, and because altered distribution of CD5+ versus CD5-negative B-cell subsets is often observed in B-cell derived disorders.

The figures on the previous page show 2 relatively similar kinetics but strikingly different profiles, as determined by CD10, CD5 and CD27 expression. The indication for rituximab therapy in patient 1 was EBV-induced lymphoproliferative disease; autoimmune cytopenias in patient 2. Despite variant immunophenotype, both patients have (so far) remained in remission from EBV disease and cytopenias, respectively.

Longitudinal determination of B-cell recovery kinetics will be tied into the phenotype of the recovering B-cell compartment, as it relates to the differentiation state of the B cells. It has been reported that the initial wave of B-cell recovery is characterized by release of naïve (transitional) recent bone marrow emigrants (as defined by expression of CD10, IgD, CD24, CD28 and lack of CD27 (identifying memory B cells). This stage in B-cell differentiation appears to be highly dependent on the BAFF system. *It is possible that recovery of memory B cells is indicative of altered B-cell reconstitution; possibly predictive of relapse of the pre-rituximab condition.*

This year, our Lab Manager, Darryl Ann Hake, celebrated her 35th year at Cincinnati Children’s Hospital. Throughout her years of service, Darryl has personified the laboratory’s mission of providing quality testing through commitment, education, and research. As a manager, she has established a corporate culture of dedication and resolute service that has set us apart from other laboratories.

Darryl began her career in 1972 as a recent graduate in the field of Medical Technology. She quickly advanced to management, and today, under her supervision, the lab has grown to a 20+ strong team of technicians and staff, performing a multitude of highly specialized assays for our regional and national clients. Darryl is considered a mentor and friend by many and we affectionately dedicate the Spring issue of the Newsletter to Darryl.
Phenotypic and functional assays pertaining to cytotoxicity are a main focus of the DIL, especially in the evaluation of hemophagocytic disorders. Insight into the cytotoxic machinery can guide the diagnostic workup and help in formulating the search for genetic disorders. Our current approach to the study of cytotoxicity combines immunophenotypic studies – including the perforin/granzyme B assay, the SAP assay and the ALPS panel – with functional assessment through the NK-cell assay and the CTL (cytotoxic T-lymphocyte) assay. In addition, several disease markers can be used to help in the measurement of disease activity and response to therapy. They include; soluble IL-2Ralpha chain (sIL-2Ra), soluble CD163, as well as plasma cytokines.

Several new projects are making their way through the R&D pipeline. In this, and the next Newsletter, an update will be given regarding 2 projects. When ready for prime-time they will become part of the “Menu”.

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Granzyme B Transfer from Effector Cells to Target Cells

GrB assay in control (top contour plots) and patient with abnormal $^{51}$Cr release assay (bottom plots). In addition to calculation of cell lysis (R3/R4) this assay can show other relevant data as well, such as conjugate formation (which could be a defective component of NK-cell function, and transfer of GrB from effector [R8] to target cells).

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**Extreme Makeover: NK-cell Edition.**

Cytotoxicity assays, like any functional lymphocyte assay consist of several components. In a simplified scheme, there is the population of cells to be studied (e.g. NK-cells, CTLs), the pathway and/or mechanism of its function (e.g. perforin/granzyme-mediated killing), a defined population of target cells (e.g. K562 cells) and a read-out method to assess the demise of the target cells. Additional complexities can be added to further determine functional characteristics (e.g. HLA blocking antibodies to interfere in MHC-restricted killing).

The component that plays a central role in the proposed Makeover is the read-out system. **Flow cytometry (FCM) will be used in parallel with the (gold standard) $^{51}$Chromium release ($^{51}$Cr) assay.** A variety of methods can be used to measure target cell killing using the flow cytometry platform (see examples in side bar on the next page).
A general principle of the FCM approach is the use of a combination of fluorescent markers; one to identify the target cell population and another to detect [a stage of] cell death. In addition to eliminating any radioactive compound, flow-based methods transform a bulk assay into an assay in which single cells are studied. The number of additional parameters that can be included in the assay, such as a more detailed characterization of the effector cell population or the visualization of conjugate formation between effector and target cells, is essentially only limited by the hardware (and software) used in the laboratory.

In our NK-cell makeover approach, killing is determined by granzyme B (GranToxiLux™ kit by OncoImmunin, Inc.) in the - fluorescence-tagged - target cells (see side box for other measurements of target cell lysis). The basic premise of this method is shown in the figures on the previous page. Upon activation of cytotoxic lymphocytes by appropriate target cells, granzyme B that is present in an inactive form in lysosomal granules, is transferred (accompanied by other granule contents such as perforin) from effector to target cells.

This process constitutes an early event in the execution of cytotoxic function that can quantitatively be measured and, when combined with the traditional $^{51}$Cr-release assay, representing the final phase of target cell lysis, provides a “from start to finish” assessment of NK-cell function. So far, we have seen good correlation between the two methods (see figure above). Interestingly, we found some discrepancies in patient samples that could be reflective of a specific issue in NK-cell function. Thus, it may be relevant to also include a FCM read-out system that measures cell death at a stage comparable to $^{51}$Cr release.

Befitting the emphasis of the DIL on disorders of hemophagocytosis, the GranToxiLux™ methodology could help in detecting disorders that are directly linked to the intracellular machinery and constituents needed for cytotoxicity. For example, defects in the transfer of granzyme B from lysosomal granules to the NK-cell membrane and subsequently to the target cells, could be detected by the retention of granzyme B in effector cells (see fig. on page 5). More on this topic in the next Newsletter (CD107; turning cytotoxic molecules inside-out).
Two new molecular diagnostic tests have been added to our armamentarium of clinical services in the Molecular Genetics Laboratory.

Familial hemophagocytic lymphohistiocytosis (FHL or HLH) is an autosomal recessive disorder of immune regulation which typically presents in early childhood and has a rapidly fatal course unless aggressively treated with chemotherapy, immune suppression and bone marrow transplant. Perforin (PRF1) and MUNC13-4 are two causative genes of familial HLH. Mutations in the PRF1 and Munc13-4 genes are identified in approximately 50-60% of individuals with familial HLH worldwide. Syntaxin-11 (STX11) is the third gene, which has been linked to familial cases of HLH. To date, mutations in STX11 have been identified in individuals of Turkish descent in which STX11 mutations may account for approximately 20% of cases of HLH. STX11 mutations have not yet been identified in Western European or other populations. NK-cell function, tested in vitro, may be normal: given the low number of HLH patients with STX11 mutations, this observation should be regarded with appropriate caution. At this time, there is no specific functional assay for the STX11 protein. Subjects with HLH, with normal PRF1 and MUNC13-4 mutational analyses are candidates for STX11 testing, particularly those from families with parental consanguinity, Turkish descent or families with multiple affected siblings.

The X-linked lymphoproliferative syndrome (XLP) is a rare, inherited immunodeficiency that is characterized by [EBV-driven] hemophagocytosis, often progressing into fatal infectious mononucleosis, hypogammaglobulinemia (common variable immunodeficiency disorder [CVID]) and lymphoproliferative disease (including lymphomas; typically of the extra-nodal non-Hodgkin variety). Disease manifestations of XLP are linked to EBV infections in about 50% of cases. Mutations in the signaling lymphocyte activation molecule (SLAM)-associated protein SAP (SH2D1A), a signaling adaptor molecule, underlie approximately 60% of cases of familial XLP.

Mutations in a second gene, BIRC4, were recently identified in an additional 20% of French patients with XLP. A thorough analysis of North American patients with a clinical phenotype consistent with XLP, but with normal SAP, is currently in progress. At this time, there is no specific functional assay for X-linked Inhibitor of Apoptosis Protein (XIAP), the protein encoded by BIRC4. Splenomegaly was noted in the few patients reported to date, while this is not a consistent feature of XLP secondary to mutations in SH2D1A. Males with clinical manifestations indicative of XLP and who have normal SAP expression or normal mutational analysis of SH2D1A are candidates for BIRC4 testing.

For further background information on HLH and XLP, please visit: www.genetest.org (search under Gene Reviews) or visit: www.cincinnatichildrens.org/dchi.

STX11/BIRC4; further reading:
These cases illustrate how HSCT can replace (components of) a defective immune system, although the specific approach of HSCT will be different in this diverse group of primary immunodeficiency disorders. For example, patients with XSCID are considerably less capable of rejecting allogeneic stem cells than patients with CGD, who have an essentially normal lymphocyte compartment (as far as rejection is concerned).

In addition to documenting correction of a defective immune system, these assays can serve as a quantitative monitor of engraftment if the assay is sufficiently sensitive in distinguishing between normal and abnormal cells (for example the detection of normal neutrophils and neutrophils with defective oxidative burst, as occurs in CGD). These figures serve as good examples of the “Before and After Principle”.

**Perforin**: detects the intracellular presence of perforin. Reduced levels are found in patients with hemophagocytic lymphohistiocytosis (HLH), on the basis of mutations in the perforin gene.

**CGD (oxidative burst)**: based on conversion of non-fluorescent dihydrorhodamine 123 into its fluorescent state following stimulation of neutrophils; a process dependent on an intact NADPH oxidase system (defective in CGD patients).
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).

**Did you know…..?**

We have launched a new service to provide our clients with easier and faster access to their patients’ results. There’s no need to wait for a faxed report; using any standard internet browser, Labtest.com enables you to view reports from the Diagnostic Immunology Laboratories the moment they are generated. For more information or to establish an account, contact our customer service representative; Julie Beach, at 513-636-4685 or julie.beach@cchmc.org

**Research Protocols**

The Immunodeficiency and Histiocytosis Program in the Division of Hematology/Oncology has a variety of research protocols. These protocols share as a main feature that they serve to better understand immunologic disease, as it pertains to increased susceptibility to infections, autoimmunity, lymphoproliferation and hemophagocytosis. These studies focus on careful characterization of clinical disease combined with comprehensive immunologic and genetic testing with the goal of improving treatment and outcome.
## CURRENT MENU OF ASSAYS

### Diagnostic Immunology Laboratory

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**From the Diagnostic Immunology Laboratories**

**Change the outcome**

**IN THE NEXT ISSUE**

- **Patient Vignette**
- **Eosinophil-based Assays (part II)**
- **More on B-cell depletion therapy**
- **CD107: Turning cytotoxicity inside-out**

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**CURRENT MENU OF ASSAYS**

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### Test Requisition Form

Visit www.cchmc.org/immunodeficiencies to view the test requisition form.

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

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### Patient Information

- **Patient Name**: [ ]
- **Date of Sample**: [ ]
- **Time of Sample**: [ ]
- **Medical Record #**: [ ]
- **Date of Birth**: [ ]
- **Sex**: Male  Female
- **Race**: American Indian  Asian  Black  Hispanic  Other  [ ]
- **Diagnosis/reason for testing**: [ ]
- **ICD-10 code**: [ ]
- **Medications**: [ ]
- **Has the patient undergone EMT?**: Yes  No  Date of EMT: [ ]

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### Test(s) being requested

**Note:** Results of a same-day CBC/Diff must accompany the sample where indicated.

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<th>Sample Requirement</th>
<th>Test Name</th>
<th>Sample Requirement</th>
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<td>Neutrophil function tests (specify from list below) **</td>
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<tr>
<td>CTL function</td>
<td>10ml (5ml) Sodium Heparin</td>
<td>Adhesion Markers **</td>
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<tr>
<td>Cytokines, Intracellular</td>
<td>3ml (2ml) Sodium Heparin</td>
<td>Phagocytosis &amp; Killing **</td>
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<tr>
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<td>Chemokines **</td>
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</tbody>
</table>

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**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 CIBP1301
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**FOCIS: Federation of Clinical Immunology Societies**

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