LYSOSOMAL STORAGE DISORDERS LINK TO AUTOPHAGY AND MITOCHONDRIAL DYSFUNCTION
By Margarita Ivanova, PhD and Ozlem Goker-Alpan, MD

The major lysosomal pathway is the cellular clearance pathway, which is the primary means for organelle turnover and clearance of unwanted proteins through proteolysis. But the role of lysosomes is not only limited to housing proteolytic enzymes: they also participate in calcium signaling, trafficking organelles, nutrient sensing, and mitochondria repair. For example, mitophagy is initiated by the damaged mitochondria itself, which is ultimately degraded by the macroautophagic pathway to compensate for energy shortage.

The degradation of dysfunctional proteins and organelles through “autophagy” is considered to be a major function of the lysosomes. Autophagy is a dynamic process, occurring in response to stress signals. One of the important stress signals for autophagy activation is environmental stimulation by for example, pharmacological agents or intercellular energy crisis due to an intracellular defect of metabolism. The key step in autophagy is the fusion of autophagic vacuoles with lysosomes to form auto phagolysosomes, where the macromolecular components are broken down into metabolites that feed into the mitochondria to provide ATP for survival. Therefore functional lysosomes are essential for autophagy, energy balance, and mitochondrial metabolism.

Lysosomal storage disorders (LSD) are a group of rare metabolic disorders caused by lysosomal dysfunction that occur due to mutations in the gene of a single lysosomal enzyme. Gaucher and Fabry diseases are the two most common inherited genetic disorders caused by deficiencies in lysosomal enzymes. Gaucher disease (GD) is caused by the accumulation of glucocerebroside in the lysosomes due to deficient glucocerebrosidase enzyme activity. Accumulation of glucocerebroside presents virtually in every cell of the organism, and symptoms may include neuropathology, skeletal disorder, enlarge spleen and liver, liver malfunction, anemia, and low thrombocyte counts. Fabry disease (FD) is an X-linked disease in which mutations of the GLA gene result in deficiency of the enzyme α-galactosidase A, (alpha–Gal A). Symptoms of FD usually appear in childhood and result in the development of life-threatening conditions such as neurodegeneration, stroke, chronic kidney disease, and heart failure.

Thus, autophagy and mitochondrial function are the logical targets to study in LSD, where the primary pathology results from lysosomal abnormalities. Our translational research center focuses on the molecular mechanisms by which lysosomes regulate autophagic and mitochondrial signaling pathways in primary cells derived from LSD patients.

Human cortical neuron cell line, Fabrazyme uptake (red)
GAUCHEROMAS: WHEN MACROPHAGES PROMOTE TUMOR FORMATION AND DISSEMINATION.
By Margarita Ivanova, PhD, and Ozlem Goker-Alpan, MD

The deficiency of the lysosomal enzyme acid β-glucocerebrosidase (GCase) and accumulation of its substrate in macrophages leads to inappropriate immune activation and dysfunction of the reticuloendothelial system organs including the liver, spleen, and lymph nodes in patients with Gaucher disease (GD). Large, foamy macrophages, known as Gaucher cells (GC), are the pathological characteristic of GD. Accumulation of large macrophages with formation of tumor-like masses, called “Gaucheroma”. Gaucheroma pose both diagnostic and therapeutic challenges, as they are resistant to available medical intervention. Gaucheromas while commonly occur in the liver and spleen, or within the bones, rarely are encountered at extraosseous sites.

We studied the pathophysiology of extraosseous Gaucheroma formation in a cohort of patients with GD. Among 63 patients with Gaucher disease followed at a single center, five patients (age range: 18-73 years) with genotypes L444P/L444P and N370S/N370S, were diagnosed with Gaucheromas, of those four had extraosseous-Gaucheromas. Among all, only one was treatment naïve. Two patients had a para-spinal mass; two others developed pelvic masses. Flow cytometry analysis from one patient, who developed T8-L12 para-spinal mass, revealed a higher expression of CCR4 cytokine on present of CD16+ non-classical monocytes in blood. On biopsy, there was infiltration of Gaucher-type cells, demonstrating reactivity against CD163, CD68 (M2 macrophage markers), and VEGF, a pro-neoplastic angiogenesis factor. However cell proliferative marker Ki67 was negative. Chemotactic factor CCL2, factor linked to augmentation of anti-tumor activity in monocytic cells was also negative. Our study indicates that extraosseous Gaucheromas are comprised of cellular elements with characteristics of tumor-associated macrophages, the major players in cancer related inflammation. The occurrence of non-classical CD16+/CCR4+ monocytes in peripheral blood may reflect the underlying cause for the accumulation of the macrophages capable of migrating to distant sites outside the reticuloendothelial system, and giving rise to tumor-like Gaucheromas. (Ivanova et al., 2016, Blood Cells Mol Dis)

Image: Gaucheroma in bones. Frontal views show multiple translucent lesions eroding the bone.
Lysosomal Storage Disorders (LSDs) are a group of over 40 inherited disorders caused by the deficiency of specific enzymes resulting in accumulation of substrates in lysosomes. There are a range of clinical symptoms manifested in various organ systems, which become progressively worse with time, often prior to testing and diagnosis. There is very limited information available about the incidence rates of LSDs in the US in general, and in African-American population in particular. While there is no data on Gaucher and Fabry disease incidence in American minorities, Pompe disease was found to be 3 times more prevalent in African-Americans compared to combined US population in one study. To bridge this gap in knowledge as well as highlight diagnostic challenges originating from ambiguous clinical manifestations, we currently perform large scale screening for ‘treatable forms’ of LSDs with a special focus on under-represented minority groups. We appreciate the grant support from Pfizer, Inc towards this study.

Under IRB approved protocols (NCT02120235 and IRB-14-MED-09), dried blood spots were prepared from left-over blood samples collected from anonymized patients who visit Howard University School of Medicine for varied health concerns. 85% of the patients are African-American and 7% are Hispanic, and the remainder report as White, other, or American Indian. The patient base consisted of almost equal distribution of males and females with their ages ranging from 15 to 100 yr. Miniaturized fluorometric enzyme assays were performed using 4-methylumbelliferyl (4-MU) substrates specific for the following LSDs.

<table>
<thead>
<tr>
<th>Enzymatic defect</th>
<th>Affected conditions</th>
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<td>α-Galactosidase</td>
<td>Fabry disease</td>
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<tr>
<td>β-Glucocerebrosidase</td>
<td>Gaucher disease</td>
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<tr>
<td>Acid α-Glucosidase</td>
<td>Pompe disease</td>
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<tr>
<td>Chitotriosidase (elevated)</td>
<td>Multiple LSDs (GD, Niemann-Pick, Krabbe, Gangliosidosis etc)</td>
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<tr>
<td>β-Galactosidase</td>
<td>Gangliosidosis</td>
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After two rounds of screening, we confirmed undetectable activity for β-glucosidase in 1 subject; α-galactosidase in 3 subjects and α-glucosidase in 1 subject. Approximately 0.05-0.1% showed significantly reduced enzyme activity, likely indicating carrier status. Interestingly, a significant number of individuals possess enzymatic activities several fold higher than the reference. These findings highlight higher incidence rates for abnormal enzyme levels in this population compared to previously published LSD screenings. The findings could be because the patient cohort was already under clinical care thus increasing the likelihood of a disease phenotype or due to ancestry-based genotype and phenotype variations. This study highlights the importance of such large scale screenings in minority groups traditionally not associated with a high incidence of LSDs. The results from the study have been presented at the following international meetings: 1) Annual meeting of American Society for Human Genetics (2015, poster #2337/T); 2) Lysosomal Disease...
Network’s WORLD symposium (2016, platform presentation as well as poster #182).

Following the hugely positive comments about our study, we were encouraged to take the study to the next level, which is to include molecular analysis for diagnostic confirmation. While enzyme assays are the first line of diagnosis, sequencing respective genes to identify causative mutations is the gold standard in assigning the disorder. In the last few months, our facility has procured Ion Torrent platform for Next-generation sequencing (NGS) with a very specific aim to design a targeted sequencing panel and develop cost effective method for validating the findings from enzyme assays. We are currently in the process of validating our custom made panel which includes primers for GBA: OMIM 606463, GAA: OMIM 606800, and GLA: OMIM 300644. The results from NGS will help in ruling out false positives and confirming the diagnosis and will present undisputable and invaluable demographics data as well as assist in offering therapy to patients with confirmed diagnosis.

GAUCHER DISEASE, ENZYME REPLACEMENT THERAPIES, AND MACROPHAGES

Margarita Ivanova, PhD

Gaucher disease (GD), one of the most common types of lysosomal storage disease, results from an inherited genetic defect that decreases, the activity of an enzyme called β-glucocerebrosidase. β-glucocerebrosidase is crucial in the maintenance of cell health via the breakdown of glucocerebroside and subsequent recycling of cellular materials in lysosomes. A deficiency in this enzyme causes an unnatural accumulation of glucocerebroside in cells, which not only hinders the normal function of individual cells, but also leads to the many symptoms observed in GD patients, such as hepatosplenomegaly, liver complications, anemia, bone abnormalities, lung disease, and even chronic neuropathic forms.

There are currently two major types of therapeutics offered to patients suffering from this disorder: enzyme replacement therapy (ERT) and substrate replacement therapy (SRT). ERT treats patients by infusing them with a functional form of the β-glucocerebrosidase enzyme to compensate for what the patient is unable to produce on his or her own, while SRT works by limiting the production and accumulation of glucocerebroside in cells. Both treatment modalities have been met with relative success. However, the efficacy and effects of both ERT and SRT are still being researched so as to provide patients with a better prognosis and quality of life.

Our lab is currently looking at the effects of these therapies on macrophages. Being that glucocerebroside accumulates most notably in macrophages, morphing them into fat-filled, misshapen foam cells called Gaucher cells, they remain one of the prime subjects of interest in GD research. Hopefully, elucidating the effects these therapies have on macrophages – their differentiation, function, and health – can lead not only to improvements in treatments for GD, but also a better understanding of the disorder overall.
Gaucher disease (GD) is caused by a genetic deficiency of the lysosomal enzyme glucocerebrosidase leading to accumulation of glycosphingolipids in various organ systems, most notably in cells of mononuclear phagocyte system. As a result, most of the studies exploring immune dysfunction in Gaucher disease have been focused on dysregulation of macrophages. Prior B-cell associated studies in GD patients have been limited to B-cell malignancies and myelomas. However, considering the fact that clinical manifestations of GD affect across organ systems, it is vital to understand possible dysregulations in various cell types in innate and adaptive immune system, i.e., T-/B- lymphocytes, NK cells and dendritic cells. We initiated a few clinical research studies involving detailed immunophenotyping of individual GD patients to investigate persistent immune alterations and their role in pathobiology of GD.

**Persistent immune alterations and effect of the delay in initiation of ERT**

The results revealed that while there are abnormalities observed in overall number of B and T-lymphocytes, NKT cells and dendritic cells are affected as well. In addition, significant differences were also seen in activated T-cells, memory markers as well as B-cell maturation, class-switching defects and development of memory B-cells are affected as well. Overall increase in CD20+/CD27+ fraction indicates that pathways lead to impaired development of memory B-cells in GD patients. IgM producing B-cells were in similar range compared to non GD controls, however there was marked alterations in IgA and IgG producing B-cells indicate persisting defects in immunoglobulin class-switching mechanism. While B-cell malignancies are definitely a concern in GD patients; our study highlights the fact that irrespective of clinical manifestations, there are multiple persistent abnormalities pertaining to immune system in GD patients. We extended the study to see the effect of delay in initiation of therapy on these long term alterations. The data demonstrates that there are different immunologic phenotypes existing in patients with Gaucher disease who are receiving ERT (Limgala et al., 2016, PLoS One). We observed that early initiation of therapy reversed most of the immune alterations as opposed to when the therapy was delayed. Patients who were diagnosed before and started therapy after the advent of ERT, thus already had significant disease load, suffering from complications, especially of skeletal in origin. Patients diagnosed after the advent of ERT were able to start receiving ERT before the severe complications ensued. Given that there are limitations of ERT for access to different tissues and organs, the outcome measures currently in use have similar limited expectations, whilst only less than half of the treated patients achieve all established treatment goals. Compared to earlier, these days there are more individuals being identified with GBA mutations at asymptomatic or minimally symptomatic stages, and more patients with GD who were able to start therapy soon after their diagnosis in childhood. There needs to be further guidelines to assess the outcome and therapeutic response as there are still co-morbidities such as hematologic malignancies resulting even in adequately treated patients. While GD is not a primary immunologic disorder, the
involvement and permanent changes in multiple immune cell lineages expand the effects of the sphingolipid metabolism and/or GBA mutations beyond the macrophage lineage.

Role of Splenectomy on long term immune effects in patients with GD

In another related but independent study, we analyzed the long term immune effects of splenectomy in patients with GD, all under ERT. Prior to the advent of ERT, therapeutic splenectomy was performed for symptomatic splenomegaly to treat the hematologic complications of hypersplenism, especially thrombocytopenia and intractable anemia. The availability of ERT reduced the incidence of splenectomy and bone disease but despite access to enzymatic and molecular diagnostic methods, splenectomy is still occasionally employed as a diagnostic modality, especially to rule out or for staging of a suspected malignancy in a patient presenting with splenomegaly and thrombocytopenia.

In order to investigate the long-term immunological effects of splenectomy, we used flow cytometry to compare the immune phenotypes of patients with GD who have undergone splenectomy (SGD) and non-splenectomized GD patients. Clinical evaluation of the patients including bone disease and other comorbidities and possible correlation with the immune findings are also elaborated. A detailed flow cytometry analysis clearly indicates major differences between the two patient populations including fewer CD27+/IgM+ B cells, more CD4+/CD45RO+ and CD8+/CD45RO+ T cells, and most importantly an almost complete absence of circulating dendritic cells in splenectomized GD patients as compared to non splenectomized GD patients. DCs are superior antigen presenting cells that capture antigens in circulation and present them to T-cells. This novel discovery of depletion of DCs in majority of SGD patients on ERT sheds new light on the immunological problems facing SGD patients. Very low levels of circulating DCs combined with low levels of CD27+/IgM+ splenic B-cells emphasize the need for routine, close monitoring of SGD patients’ health and immune system as well as the need to keep vaccines up to date (Sønder et al., 2016, Blood Cells Mol Dis).