Investigations on the role of impaired lysosomes of macrophages in disease

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Chapter 7

Discussion and summary

Discussion

Historical and renewed interest in lysosomes

Since the seminal studies by De Duve and co-workers in the late fifties, cells are known to contain 100-200 distinct membrane enclosed acid compartments that are named lysosomes ('bodies of cleavage').[1] These organelles are responsible for fragmentation of intra- and extracellular macromolecules that enter lysosomes via endocytosis, macropinocytosis or autophagy. Fragmentation relies on the catalytic action of hydrolases that are assisted by accessory proteins and low luminal pH. The surrounding membrane of lysosomes is equipped with transporters that assist in export of degradation products, ion-channels and the v-ATPase that maintains the acid pH at the expense of ATP.[2] The interest in lysosomes was boosted in the sixties by the identification of genetic disorders that result in deficiencies in lysosomal hydrolases or supporting proteins.[3,4] Since in most of these disorders the substrate of the deficient enzyme accumulates, inherited lysosomal deficiencies became collectively named as lysosomal storage diseases (LSDs). Presently, over 60 LSDs are known and also include genetic defects in non-enzymatic constituents of lysosomes such as transporters, activator proteins and other protein factors.[5] In the case of inherited lysosomal enzymopathies, the corresponding enzymes were purified and characterized in the eighties and for most of these LSDs, the deficient gene has meanwhile been cloned. These developments revolutionized laboratory diagnosis and initiated research on therapies for lysosomal enzymopathies. Instrumental in this regard was the gained knowledge of biosynthesis, intracellular sorting and lectin-mediated uptake of lysosomal enzymes.[6] From the late seventies onwards, attempts were made to treat lysosomal enzymopathies by administration of the lacking hydrolase: the breakthrough was enzyme replacement therapy (ERT) for type 1 Gaucher disease through chronic infusions with a mannose-terminated glucocerebrosidase ensuring lectin-receptor uptake by tissue macrophages.[7] Such ERT was soon copied for other lysosomal enzyme deficiencies like Fabry disease and Pompe disease, albeit with limited clinical success. In the last two decades alternative therapies for some LSDs have been designed based on small compound drugs, e.g. substrate reduction therapy (SRT) and enzyme stabilizing chaperone therapies.[8,9] At present, vast effort is paid to a variety of gene therapy approaches employing viral vectors (conventional gene therapies, RNA therapies and siRNA therapies).[10,11] Interest in the cellular role of lysosomes was boosted by the appreciation of a key role for autophagy in maintenance of cell integrity, culminating in the Nobel prize that was recently awarded to Yoshio Oshumi.[12] In this millennium lysosome research got further, major momentum by the realization that the organelles not merely play a role in macromolecule fragmentation but are actively involved in the regulation of key cellular processes.[13] It was discovered that two key regulatory kinases in cells, mTORC1 (master regulator of cell growth and metabolism mechanistic target of rapamycin [serine/threonine kinase] complex 1) and AMPK (AMP-activated protein kinase), can be associated with the surface of lysosomes and as such link metabolite supply from lysosomes with processes like cell growth and metabolism.[13] Moreover, it has become apparent that mTORC1 regulates the activity of transcription factors of the MiT/TFE (microphthalmia-transcription factor E) family, a group of transcription factors that promote the expression of genes encoding lysosomal proteins and autophagy

components.[14] In this way, supply of metabolites from lysosomes is intricately linked to *de novo* formation of lysosomes, autophagy and endocytosis. Knowledge on membrane proteins of lysosomes has been lagging for some time, but their composition on the lysosomal membrane surface and their functions is increasingly elucidated.[15,16] Similarly, regulation of the membrane content of cells has been limitedly understood, but again crucially involves lysosomes. Turnover of membranes in cells takes place in lysosomes following formation of multi-vesicular bodies generated in endosomes as well through delivery via autophagy. By means of fission and fusion, lysosomes and endosomes dynamically interact with each other, as well as with the plasma membrane through endocytosis and phagocytosis. More recently, the relevance of exocytosis of lysosomes has begun to be appreciated.[17] In specialized cells, lysosomes fulfil specific functions, attracting further attention of researchers. Examples of this are immune cells that phagocytose pathogens, antigens, senescent and apoptotic cells as well as osteoclasts involved in bone remodelling. In view of their amazingly broad functions lysosomes presently receive interest in various fields such as inherited and acquired metabolic disorders, infectious diseases, neurodegenerative diseases, cancer, and ageing.[18–20]

This thesis aims to elucidate several aspects of lysosomes and their outcome is here further discussed.

Chapters 1 and 2 deal with perturbation of **lysosome function in general and that of lysosomal enzymes in cultured cells**. Cultured cells, and fibroblasts in particular, have been amply used as the biochemical confirmation of diagnosis of LSDs. In addition, they have been employed as models to study the biosynthesis, intracellular transport and uptake of lysosomal enzymes. The importance of the acidic lysosomal pH has also been elucidated by use of cultured cells. Lysosomotropic weak bases such as methylamine and chloroquine were used to increase endosomal and lysosomal pH and thus disrupt lysosomal processes in cultured cells. The mannose-6-phosphate receptor mediated sorting of newly formed lysosomal enzymes relies on low pH for the release of enzyme ligands from the receptors. Consequently, the presence of lysosomotropic weak bases in the cell culture medium also interferes with delivery of acid hydrolases to lysosomes.[21,22] The effects of lysosomotropic bases illustrate that lysosomes of cultured cells can be markedly influenced by the culture medium composition.

Chapter 1 addresses the **impact of the presence of the zwitterionic buffer HEPES in cell culture medium on cultured cells**, nowadays a popular addition to stabilize medium pH. HEPES as buffer in biological systems was originally described by Good and co-workers. It was found to exert little effect on isolated mitochondria and purified bacterial enzymes.[23] Later studies reported no toxic effects of the presence of HEPES in cell culture medium and even noted an increase in growth rate as compared to bicarbonate supplemented medium.[24] However, as described in chapter 1, the presence of HEPES (25 mM) in culture medium does significantly influence lysosomes of cells. Since HEPES is a potent buffer with a pKa of 7.4, chronic exposure of cells to HEPES likely results in elevated lysosomal pH which impairs lysosomal function. A similar phenomenon is triggered by chloroquine and the v-ATPase inhibitor bafilomycin. Increased lysosomal pH is well known to reduce the activity of numerous lysosomal hydrolases and thus disturb

metabolic fluxes in cells.[25] In addition, membrane flow in the endolysosomal apparatus is impaired by pH changes induced by permeable weak bases and various low pH-dependent receptor-mediated endocytic mechanisms are reduced at such conditions.[26] It has been recently reported that perturbed lysosomal pH induces a rescue mechanism in which interaction of the STAT3 (Signal transducer and activator of transcription 3) protein with the ATP6V1A subunit of the lysosomal v-ATPase promotes reduction of lysosomal pH. Acidification of the cytosol or alkalinization of the lysosomal lumen triggers STAT3 to the lysosome, at the expense of STAT3-mediated transcription.[27]

Exposure of cells to HEPES induces the translocation of MiT/TFE transcription factors, in turn promoting lysosome biogenesis and autophagy. This response likely constitutes an attempt to restore the catabolic flux in cells with impaired lysosomes. Remarkable is the finding that the MiT/TFE translocation upon HEPES stimulation proceeds independently of mTORC1, as phosphorylation of its prime substrates remained unaltered in HEPES exposed cells.[28] It therefore poses a paradox with respect to mTORC1 as a master regulator of anabolism.[13,29,30] Indeed, MiT/TFE members are reported to be activated by several other proteins, including PKC, PKD, GSK3β and phosphatases, and further research on cell specificity and redundancy of these pathways in regulating MiT/TFE members is required.[31–35]

Chapter 2 illustrates how the **lysosomal enzyme glucocerebrosidase (GCase) acts as sensitive indicator for disturbances in lysosomes induced by the presence of HEPES in the culture medium**. GCase fundamentally differs from other soluble lysosomal hydrolases: it is not sorted to lysosomes via mannose-6-phosphate receptors, but it binds to the membrane protein LIMP2 in the endoplasmic reticulum soon after folding. As complex, LIMP2 and GCase are sorted to lysosomes where low local pH triggers dissociation. GCase is a glycoprotein containing 4 N-linked glycans that are largely converted in the Golgi apparatus from high mannose-structures to sialylated complex-type ones. These modifications are reflected by an increase in molecular weight of 62 kDa (ER) to 66 kDa (trans-Golgi). Upon delivery into the lysosome, exoglycosidases gradually trim the glycans of GCase resulting in stepwise formation of a 'mature' 58 kDa form of the enzyme. As such, the precise glycan composition of GCase does not influence its enzymatic activity.[36] Inside the lysosome, GCase is also subjected to proteolytic breakdown that is inhibitable by leupeptin. Exposing cultured cells to HEPES markedly reduces the maturation of 66 kDa GCase and its proteolytic degradation: accumulation of 66 kDa enzyme in less dense organelles occurs under these conditions. This is likely caused by a disturbed intralysosomal milieu that inhibits glycosidases and proteases. Since GCase turnover in lysosomes is relatively fast, a reduction in its intralysosomal degradation as induced by HEPES leads to a marked increase in cellular content on active enzyme molecules. This can be visualized with selective activity-based probes for GCase (see section below) and the measurement of enzyme activity in cell lysates. In other words, cells cultured in the presence of HEPES tend to show higher cellular GCase levels than when grown without buffer in the culture medium. This phenomenon is relevant for the enzymatic diagnosis of Gaucher disease with cultured cells as source of enzyme. A negative impact of the presence of HEPES in the culture medium is also observed for other lysosomal hydrolases such as acid alpha-glucosidase and beta-glucuronidase. This highlights that caution is warranted when interpreting data on lysosomes of cells that

are cultured in the presence of HEPES. Recently, GCase specific substrates have become available that can measure enzyme activity inside lysosomes of cultured living cells, providing alternative ways to measure GCase activity.[37]

Besides accumulating protons, lysosomes also act as store for Ca^{2+} ions. Lysosomal Ca^{2+} concentration can be as high as 0.5 mM. Released Ca^{2+} from lysosomes is postulated to act as second messenger that activates calcium dependent cellular processes.[38–40] TRPML1 (Mucolipin TRP channel 1) mediated calcium efflux from lysosomes has been linked to activation of the phosphatase calcineurin and subsequent dephosphorylation of TFEB (transcription factor EB).[41] It is presently unknown whether the presence of HEPES in the culture medium impacts on lysosomal Ca^{2+} concentration besides H^+ concentration, and whether lysosomal Ca^{2+} release contributes to mTORC1-independent activation of MiT/TFE transcription factors. Lysosomal calcium is also crucially involved in the fusion of lysosomes with other organelles, as well as in lysosome exocytosis. SNAREs (Soluble N-ethylmaleimide-sensitive factor attachment protein receptors) form a parallel four-helix bundle called a *trans*-SNARE complex in a Ca^{2+}-dependent manner. This facilitates the merger of opposing membranes and exchange of content.[42]

Chapter 3 focusses attention to the **composition of individual lysosomes in cells**. It has become clear that cellular lysosomes (>100) are heterogeneous of nature. Individual lysosomes in cells move inward (retrograde; towards the microtubule-organizing center (MTOC)) and outward (anterograde) along microtubules, assisted by adaptor proteins (dynein and kinesin, respectively).[43] The perinuclear lysosomes are found to be on average more acid and catabolically active than the peripheral located organelles.[44] During nutrient excess, anterograde outward movement is more frequent, whilst during nutrient shortage retrograde inward transport is more prominent. It is presently unknown whether the hydrolase composition of all lysosomes is similar. The recent design of cyclophellitol-based activity probes (ABPs) for various retaining glycosidases has led to tools allowing selective labelling of alpha- and beta-glucosidase, alpha- and bet -mannosidases, alpha- and beta-galactosidases, beta-glucuronidase, alpha-iduronidase and alpha-fucosidase.[45] Fluorophore-tagged ABPs allow labelling of corresponding active glycosidases and an unprecedented visualization of them in living cells. Chapter 3 describes the fluorescent labelling of active GCase molecules in intact fibroblasts. Correlative light and electron microscopy (CLEM) allows visualization of labelled GCase molecules in individual electron dense lysosomes. Labelling therapeutic enzyme with a different fluorophore-tagged ABP allows simultaneous detection of endogenous GCase and exogenous enzyme in mannose-receptor expressing cells exposed to the therapeutic agent. The study revealed that the majority of individual lysosomes was supplemented with therapeutic enzyme. The finding provides an explanation for the clinical success of enzyme replacement therapy of Gaucher disease and suggests the exchange of content among lysosomes. The CLEM technology could in principle employed to visualize multiple lysosomal glycosidases for the study of uniformity of lysosomes in cells exposed to various conditions (e.g. nutrients, hormones, lysosomotropic agents, hydrolase inhibitors). Another future application for ABPs is labelling of therapeutic recombinant enzyme with suitable tagged probes, followed by infusion and subsequent monitoring of tissue distribution with non-invasive scanning techniques, for example by PET-SCAN.[46]

Chapters 4 and 5 are studies on **storage cells encountered in LSDs**. In many LSDs, tissue macrophages transform into storage cells. The particular vulnerability of these cells is not surprizing given their role in ongoing phagocytosis and endocytosis of senescent and apoptotic cells as well as lipoproteins. These pathways imply high rates of lysosomal degradation of substrates and handling of products. Particularly in sphingolipid storage disorders the presence of lipid-laden macrophages is very prominent: examples of this are Gaucher cells in Gaucher disease (glucocerebrosidase deficiency) and Pick cells in Niemann-Pick diseases types A and B (acid sphingomyelinase deficiency). Factors that are produced by storage cells and released into the circulation have been actively searched for since these may be employed to assist diagnosis, to monitor progression of disease and to capture corrections by therapeutic interventions. At the forefront in this respect has been the detection of biomarkers for Gaucher disease, i.e. factors released by the glucosylceramide-laden macrophages. It was discovered that these Gaucher cells produce and secrete high quantities of the chitinase chitotriosidase and the chemokine CCL18, leading to strikingly elevated plasma levels in symptomatic patients, on average 1000-fold and 40-fold respectively.[45] Increased plasma levels of chitotriosidase are encountered in various LSDs, illustrating the common involvement of macrophages in storage accumulation in these disorders.[47] In many centres world-wide, plasma chitotriosidase measurement is nowadays employed as a first screen for the potential existence of a lysosomal storage disorder in a suspected individual.

Chapter 4 reviews the knowledge on **glycoprotein non metastatic protein B (GPNMB)**, a more recently identified marker for lysosomal dysfunction. GPNMB is a 110 kDa membrane protein that is selectively expressed, most prominently in melanocytes and in phagocytes subjected to lysosomal stressors.[48] GPNMB is excessively produced by Gaucher cells in spleen of GD patients, and remarkably also by RAW264.6 cells exposed to HEPES, a relative mild stressor of lysosomes. The protein has been reported to co-localize with phagosomes and lysosomes.[49] A soluble fragment of GPNMB is released by Gaucher cells, most likely via cleavage by ADAM10.[50,51] The mechanism of active GPNMB shedding explains the more than tenfold elevated plasma levels encountered in symptomatic GD patients. The precise function of GPNMB and its soluble fragment in context of lysosome perturbation has still not been established.[48] It is presently thought that induction of GPNMB expression occurs in response to lysosomal storage, in particular through stress by excessive lipids. Consistent with this is the increased expression of GPNMB in lipid-laden macrophages of GD patients and in macrophages of patients and mice with Niemann Pick disease type C (NPC) showing lysosomal accumulation of sphingolipids and cholesterol resulting from impaired export of the sterol.[52–54] In addition, elevated GPNMB has also been observed in acquired metabolic disorders characterized by lipid-stressed macrophages such as atherosclerosis and obesity.[54,55] Finally, microglia, the resident brain macrophages, can also excessively produce GPNMB as was discovered in multiple sclerosis and neuronopathic Gaucher mice.[56,57] It is presently unclear whether the upregulation of GPNMB serves some protection for stressed lysosomes. GPNMB has been described to mediate LC-3 dependent phagocytosis in macrophages, a process that utilizes aspects of the autophagy machinery.[49] Possibly, the increased intracellular GPNMB following perturbation of lysosomes reflects adaptations in the endolysosomal apparatus. GPNMB expression is known to be controlled by MITF, a member of the MiT/TFE transcription

factor family, further hinting to a role in the endolysosomal apparatus.[54,58] In fact, the physiological relevance of the marked upregulation of chitotriosidase and CCL18 in Gaucher cells is neither identified yet.

Based on amino acid homology, GPNMB has been earlier proposed to contain a lectin-binding domain with some affinity to galactose structures.[59] Of interest, a genuine galactose-lectin, galectin 3, is also found to be modestly increased in GD patients, NPC mice, and individuals suffering from obesity, atherosclerosis, α-synucleinopathies, atherosclerosis and obesity.[60–63] Galectin-3 has been shown to be involved in repair of lysosomal membrane permeabilization.[64,65] In addition, it has been associated with membrane repair involving autophagy.[65,66] Extracellular galectin-3 has been proposed to play a role in intercellular communication and was found to be associated with insulin resistance during obesity.[61]

Adaptations to lysosomal defects is characterized in *chapter 5*, which focusses on the liver of mice that are deficient in the cholesterol efflux transporter Niemann-Pick type C1 ($NPC1^{-/-}$). In certain LSDs, it has become apparent that a blockade in lysosomal catabolism due to an impaired hydrolase results in alternative metabolism. An example of this is offered by Gaucher disease. The deficiency of GCase causes lysosomal accumulation of glucosylceramide (GlcCer), but part of the lipid is converted by acid ceramidase to glucosylsphingosine (GlcSph).[67] The latter lipid is water-soluble and may leave lysosomes, cells and even the body via bile and urine. Similarly, alternative lysosomal deacylation of accumulating glycolipids occurs in other LSDs, e.g. formation of lysoGb3 (globotriaosylsphingosine in Fabry disease) and galactosylsphingosine in Krabbe disease.[45] Elevated deacylated sphingolipids (lyso-sphingolipids) are considered to be toxic. For example GlcSph has been proposed to induce osteopenia, α-synuclein aggregation, gammopathy and related multiple myeloma and to activate the complement cascade.[45] Likewise, lysoGb3 has been proposed to contribute to podocyturia, fibrosis and loss of nociceptive neurons in Fabry disease patients.[45] Finally, excessive galactosphingosine (originally named psychosine) in neurodegenerative Krabbe disease is thought to be neurotoxic.[45]

An additional consequence of lysosomal enzyme deficiency can be the redistribution of substrate and altered destination of products. For example, the activity of the cytosol-facing GCase homologue glucosylceramidase (GBA2) is increased in GCase-deficient cells, along with extralysosomal GlcCer degradation and a concomitant formation of glucosylated cholesterol (GlcChol) via so-called transglucosylation.[68] A particularly intriguing LSD in this respect is NPC, which manifests with a primary lysosomal cholesterol accumulation but is accompanied by secondary accumulation of sphingomyelin and GlcCer. In addition, NPC patients show elevated levels of GlcChol that is formed by the transfer of the glucose moiety from GlcCer to cholesterol in lysosomes by GCase.[68] The modestly elevated GlcSph in NPC deficient patients and mice is likely caused by increased deacylation of excessive GlcCer, as in GD.[69]

In *chapter 5,* the status of GCase was investigated in livers of mice lacking NPC1 protein. In the studied NPC livers, the most prominent pathological hallmark was the acquisition of characteristic lipid-laden storage macrophages. However, an altered ultrastructural appearance was also observed for hepatocytes. In total liver of NPC mice, a reduced enzyme activity as well GCase protein content was observed. In contrast, GBA2 levels tended to be

inversely correlated to GCase deficiency. There was variation among individual mice in the extent of the effect, but the changes in GCase and GBA2 levels were always reciprocal. Next, immunohistochemistry was used to study lysosomes in hepatocytes and Kupffer cells (resident macrophages) of 80 weeks old murine NPC liver. The investigation pointed to fundamentally distinct adaptations in hepatocytes compared to Kupffer cells. Increased levels of GPNMB, galectin-3 and the lysosomal protease cathepsin D were observed in the Kuppfer cells, but not hepatocytes. In striking contrast, the hepatocytes in the NPC1-deficient liver showed a marked increase in LIMP2, a phenomenon not observed for the lipid-laden Kupffer cells. Recently it has been recognized by crystallography that LIMP2, the transporter of newly formed GCase to lysosomes, harbours a channel structure. Given structural similarity of LIMP2 with CD36, it is presently proposed that LIMP2 might act as a transporter for cholesterol.[70] Indeed, it has been observed that LIMP2 might assist export of cholesterol from lysosomes, as indicated by the finding that a double deficiency in NPC1 and LIMP2 results in a more prominent SREBP2-driven induction of HMGCoA reductase transcription, a classic readout for impaired cholesterol efflux from lysosomes.[71] Based on these findings it seems conceivable that LIMP2 may facilitate transport of cholesterol from the lysosome when the regular NPC1-mediated pathway is absent. In theory, it cannot be excluded that LIMP2 might act as transporter of GlcChol. Depending on metabolite concentrations, GCase is known to be able to either catabolize GlcChol to glucose and cholesterol or to generate GlcChol from GlcCer and cholesterol. Moreover, the catalytic pocket of GCase bound to LIMP2 is relatively close to the presumed cholesterol channel of LIMP2. Of further note, NPC patients are presently treated with Miglustat (N-butyldeoxynojirimycin), a potent inhibitor of GBA2, the primary enzyme forming GlcChol in cells. The relevance of this with respect to export of cholesterol (or GlcChol) from lysosomes via LIMP2 is not established.

The location of LIMP2 in hepatocytes tends to be largely confined to the peribiliary region of the cytosol suggesting overlap with previously described location of lysosomes.[72,73] Since lysosomes may excrete cargo into bile by exocytosis, we consider the possibility that the increased abundance of LIMP2 in NPC hepatocytes telltales compensatory facilitation of cholesterol export into bile canaliculae.[74] In absence of NPC1, hepatocytes may thus be protected from cholesterol excess by LIMP2 upregulation.

Presently, NPC receives attention from many different research fields: researchers with an interest in cellular cholesterol homeostasis, investigators of lysosomal storage disorders and neuroscientists with an interest in degenerative conditions such as Alzheimer's disease.[75–77] This illustrates the enormous cellular and physiological implications of a relatively simple monogenetic defect involving lysosomes.

Chapter 6 deals with **the role of MiT/TFE transcription factors in lysosomal biogenesis** in macrophages residing in adipose tissue of obese mice. The MiT/TFE subfamily of basic helix-loop-helix (bHLH) transcription factors consists of TFEB, TFE3 (transcription factor 3), MITF (melanogenesis associated transcription factor) and TFEC (transcription factor EC).[78] TFEB was the first to be identified as binding to a common sequence upstream of lysosomal genes, the so called Coordinated Lysosomal Expression and Regulation (CLEAR) element.[79] TFEB and TFE3 are ubiquitously expressed, whereas MITF is largely restricted to pigmented cells such as melanocytes and retinal epithelium cells, as well as myeloid cells of the immune system, osteoclasts, and stem cells of the hair

follicle.[78,80] TFEC has been shown to be strictly expressed in macrophages.[81] TFEB, TFE3 and MITF undergo phosphorylation by mTORC1, the master regulator of cell growth that resides at the cytosolic side of the lysosomal membrane.[13,82–84] It is still unknown whether TFEC is also modified by mTORC1. Under basal, nutrient rich, conditions, heterodimeric RAG GTPases recruit mTORC1 to the lysosome, thereby facillitating its interaction with the activator protein RHEB (Ras homologue enriched in brain). Active mTORC1 phosphorylates a serine residue (Ser^{211}) of TFEB that mediates cytosolic retention by 14-3-3 proteins.[85,86] Likewise, the phosphorylation of a homologous serine (Ser^{173}) in MITF governs its cytosolic retention.[87] Inhibition of mTORC1 results in dephosphorylation of TFEB, MITF and TFE3 and their nuclear localization. Selective inhibition of mTORC1 by Torin 1 and lysosomal amino acid starvation promotes translocation of TFEB to the nucleus.[79,85,88,89] Stressors of lysosomes such as the undegradable sucrose or lysosomotropic chloroquine induce the nuclear translocation of TFEB, MITF and TFE3.[28] During lysosomal deficiencies, increased transcriptional activity of TFEB and other TFs has been observed.[79,90–93]

Transcriptional activity of MiT/TFE factors is complex. Homodimerization and heterodimerization among the MiT/TF factors have been described.[78,94] The cell specificity of these TFs and their posttranscriptional (splicing) and posttranslational (phosphorylation, acetylation) processing impacts on transcriptional action.[85,95,96] In bone marrow derived monocytes, MITF and TFE3 have been reported to be phosphorylated upon M-CSF (macrophage-colony stimulating factor) stimulation via ERK1/2 (extracellular-signal regulated kinase 1/2) / MAPK (mitogen-activated protein kinase).[97] Of note, phosphorylation of TFEB by ERK2 was found to result in cytosolic retention of TFEB.[88] Besides ERK1/2, MITF has also been reported to be serine-phosphorylated by the p38 MAPK.[81,98] Moreover, the PI3K/AKT route has been described as an alternative pathway for activation of MITF in myeloid cells. IL-10 stimulation of dendritic cells causes nuclear translocation of MITF, triggering GPNMB expression through inhibition of PI3K/AKT and subsequent activation of GSK3β.[58,99] GSK3β is thought to phosphorylate MITF on Ser^{298} and thereby allows MITF to transactivate promotor regions of target genes. Natural substitution of Ser^{298} in MITF to proline strongly affects binding affinity to consensus DNA target motifs and gives rise to Waardenburg Syndrome type 2 , a syndrome characterized by a lack of skin pigmentation.[99,100] Recently, acetylation has been added to the possible posttranslational modifications of MiT/TFE members. For example, MITF was shown to be subjected to MAPK/p300 dependent acetylation in melanocytes changing binding affinity to DNA-regions.[96]

Other levels of regulation of MiT/TFE mediated transcription have become apparent. Mass spectrometry analysis of the nuclear binding partners of MITF has revealed an extensive interactome.[101] Furthermore, recent studies point to a role of chromatin modifications in MiT/TFE mediated lysosomal biogenesis.[102] In addition, HDACs (histone acetylases or deacetylases), particularly HDAC2, facilitate binding of c-Myc to the promotors of genes encoding lysosomal proteins, thus competing their MiT/TFE mediated transcription.[102] Zkscan3 (zinc finger with KRAB and SCAN domains 3) has been proposed to act as a repressor of lysosomal biogenesis, likely by competing MiT/TFE mediated transcription.[31,103] Thus, the chromatin landscape and other TFs may further regulate MiT/TFE mediated transcription.

The role of MiT/TFE in pathologies characterized by lipid-laden cells such as macrophages remains incompletely understood. Obesity is generally associated with a combination of pathologies (insulin resistance, hypertension, hypercholesterolemia and hypertriglyceridemia) that are classified as the metabolic syndrome.[104,105] Obese adipose tissue tends to be inflamed and a macrophage orchestrated low-grade inflammation is generally considered to drive insulin resistance in diabetic obese individuals. It is assumed that communication between neighbouring macrophages with adipocytes in obese adipose tissue occurs. On the one hand, macrophages are involved in phagocytic clearance of apoptotic adipocytes, visible as so-called crown-like structures.[106,107] On the other hand, macrophages respond to factors released by adipocytes, for example adiponectin, a hormone that sensitizes tissues for insulin and alleviates lipotoxicity and inflammation.[108] Xu et al. noted that obese adipose tissue macrophages (ATMs) have a foamy appearance suggesting lysosomal lipid stress.[109] In line with this, GPNMB, a marker for perturbation of lysosomes in macrophages, is dramatically increased in obese mice and, to a lesser extent, in men.[48,54] In cultured macrophage-like RAW264.7 cells, MITF-mediated GPNMB expression is induced by feeding palmitate and chloroquine.[54]

Given the previous findings regarding MiT/TFE driven induction of the lysosomal apparatus in macrophages upon lysosomal stress, we investigated the role of these transcription factors in macrophages residing in adipose tissue of obese mice. The key objective was to elucidate whether the MiT/TFE mediated response in the phagocytes is beneficial or harmful. To study this, use was made of siRNAs targeting MiT/TFE transcription factors and their selective delivery to macrophages in adipose tissue (ATM) through encapsulation in glucan particles (Gerps).[110]

First, the siRNAs targeting *Mitf, Tfeb* and *Tfe3* were examined regarding efficacy using cultured RAW264.7 cells. Using *Gpnmb* expression as readout, only simultaneous siRNA knockdown of *Mitf, Tfeb* and *Tfe3* mRNA resulted in marked reduction of *Gpnmb* mRNA. Next, mice were intraperitoneally treated with Gerps containing a mixture of siRNAs targeting the three MiT/TFE family members or Gerps containing scrambled siRNA2. After two weeks, the animals were sacrificed, and tissues and blood were collected for analyses. No significant reductions were observed in adipose tissue of mice treated with Gerps containing MiT/TFE siRNAs with respect to transcription factor mRNA levels and *Gpnmb* expression. However, there was marked variation among individual mice in both groups (Gerps with MiT/TFE siRNAs and those with scrambled siRNAs). Next, $CD11b^{+}$-cells were isolated and examined, revealing again no significant changes in transcription factor mRNA levels and *Gpnmb* expression. In $CD11b^{+}$-cells from MiT/TFE siRNA treated mice only *Cd9* and *Lpl* mRNA levels were found to be significantly reduced. CD9 is a tetraspanin that is associated with exosomes and is implicated in several biological processes including reproduction.[111,112] Increased expression of *CD9* and *LPL* mRNA has been found to be accompanied by increased expression of *TREM2* in macrophages (or migroglia) cells.[113,114] In obese adipose tissue *TREM2* positive, lipid laden macrophages have been identified by single-cell sequencing.[113] Obese *Trem2* knock-out mice manifest with dramatically increased adipocyte size and worsening of whole-body metabolism compared to obese wild type mice.[113] Upon administration of MiT/TFE-siRNA Gerps, *Trem2* levels were found to be significantly lower in epididymal white adipose tissue. The finding that *Gpnmb* expression was not prominently reduced in macrophages in adipose

tissue of MiT/TFE-siRNA Gerp treated obese mice is remarkable. Nevertheless, the treatment resulted in reduced glucose clearance in an intraperitoneal glucose tolerance test, suggesting increased insulin resistance. Moreover, the treated mice showed a reduced adiponectin expression in their adipose tissue. The reduction in adiponectin expression in adipose tissue is relevant since this adipokine is known to act as potent insulin sensitizer and protector against lipotoxicity.[108] The mechanism by which MiT/TFE suppression in macrophages effects adipocytes (adiponectin expression) is unknown.

It should be kept in mind that other transcription factors directly, or indirectly (c-Myc), play a major role in regulation of lysosomal biogenesis. Besides the MiT/TFE transcription factors, STAT3 has recently been reported to drive transcription of some lysosomal genes.[115] Of note, STAT3 shares a 50 amino acid motif with MITF that binds PIAS3 (protein inhibitor of activated STAT3), suggesting common regulation.[116,117]

Future questions

The described PhD investigations concerned lysosomes, in particular those in macrophages. Macrophages are well known to be heterogenous and generally two major phenotypes are distinguished: the inflammatory M1-macrophages and the alternatively activated M2-cells.[118] It is assumed that lysosomal lipid accumulation promotes the differentiation of macrophages to the M2-phenotype, for example in spleen and liver of Gaucher disease patients and in adipose tissue of obese individuals (described in this thesis).[119] A similar M1/M2 distinction is also made for the heterogeneous microglia in the brain.[114,120] In general, M1-phagocytes are thought to promote pathology due to their inflammatory nature, whereas M2-cells dampen such processes and might exert beneficial roles in whole body metabolism in atherosclerosis and during obesity.[113,121–123] It will be of interest to establish whether differences exist in lysosomal biogenesis and lysosome composition of M1-macrophages and their M2-counterparts. It has been reported that active mTORC1 promotes inflammatory M1-macrophages, whereas in M2-macrophages, TFEB transcriptional activity is high.[124,125] In monocytes, MITF was shown to drive GPNMB expression as part of differentiation towards a M2-like, T-cell suppressive, phenotype.[58] Conceivably, and testable in future research, the M2-phenotype of tissue residing macrophages may be related to MiT/TFE transcription. If so, beneficial M2-macrophage differentiation could be promoted by (modest) lysosomal stress and/or lysosome perturbation.

Storage cells in inherited LSDs have been historically viewed as the major culprits in pathology. However, they might also modulate pathological processes, e.g. by containing toxic metabolites in storage material and through anti-inflammatory action. The occurrence of perturbed lysosomes not only has negative consequences for cells and their surroundings. It may lead to favourable induction of lysosomal biogenesis, promotion of autophagy and synthesis of anti-inflammatory cytokines. In other words, lysosomal accumulation of lipids in macrophages ('storage cells') within a tissue might give rise to various responses, including even beneficial ones. In view of this consideration, correcting the lysosomal storage in lipid-laden macrophages, present in inherited and acquired disorders and increasing with ageing, may render unforeseen outcomes.

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