IMPLICATION OF THE DISEASE-ASSOCIATED *ORMDL3* IN MACROPHAGE PHYSIOLOGY

KERSTIN KIEFER — TESI DOCTORAL UPF / 2016

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Für meine Eltern,

Susanne und Gerd

und meine Großeltern.

It's good to have an end to journey toward,
but it is the journey that matters in the end.

Ernest Hemingway

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Abstract

Genome-wide association studies linked increased *ORMDL3* expression levels to several inflammatory diseases via single nucleotide polymorphisms. However, the pathophysiological mechanisms underlying this association are still poorly understood. ORMDL proteins have been shown to regulate *de novo* sphingolipid synthesis among other functions, a process important for proper innate immune responses and its imbalance was furthermore associated to asthma disease. This thesis was aimed to provide further inside into the ORMDL-SPT complex formation and to elucidate potential involvement of ORMDL3 in macrophage physiology using a transgenic mouse model. We herein demonstrated that a structural rearrangement under high sphingolipid content takes place, in order to release SPT activity. Moreover, ORMDL3 levels decreased cellular ceramide content in macrophages, and specifically impaired the *de novo* synthesis during activation leading to reduced autophagy and bacterial clearance. In addition, we explore the SNP-dependent regulation of *ORMDL3* gene expression in human monocytes. Taken together, this thesis contributes to a better understanding of the implication of ORMDL3 in sphingolipid homeostasis and underlines its importance in macrophage physiology.

Resum

Alguns estudis d'associació genòmica han relacionat un augment dels nivells d'expressió d'ORMDL3, causat per polimorfismes d'un sol nucleòtid, amb diverses malalties inflamatòries. Tot i així, els mecanismes fisiopatològics subjacents a aquesta associació no són del tot coneguts. Les proteïnes ORMDL estan involucrades en la síntesi d'esfingolípids *de novo*, un procés essencial per a la resposta immunitària de tipus innat i que ha estat associat amb l'asma. Aquesta tesi té l'objectiu d'aprofundir en la formació del complex ORMDL-SPT així com d'elucidar la potencial implicació d'ORMDL3 en la fisiologia dels macròfags utilitzant un model de ratolí transgènic. Els nostres resultats demostren un rearranjament estructural quan hi ha un alt contingut d'esfingolípids, per tal d'alliberar l'activitat de l'SPT. A més, els nivells d'ORMDL3 disminueixen el

contingut cel·lular de ceramides i afecten específicament la síntesi *de novo* durant l'activació dels macròfags, produint una reducció en l'autofàgia i la liquidació bacteriana. Addicionalment, hem explorat la regulació d'ORMDL3 depenent de polimorfismes en monòcits humans. En conjunt, aquesta tesi contribueix a un coneixement més profund de la implicació d'ORMDL3 en la homeòstasi d'esfingolípids i remarca la seva importància en la fisiologia dels macròfags.

Prologue

Genome-wide association studies are a powerful tool to elucidate genetic causes for complex diseases, since the identification of variation in individual nucleotides with a high allelic frequency found in the disease often leads to the discovery of new candidate genes. Following this GWAS approach, the SNP rs7216389 was strongly and reproducibly associated to the risk of developing asthma disease and other inflammatory diseases, such as inflammatory bowel disease, rheumatic arthritis and diabetes type I, which came along with increased *ORMDL3* expression levels. This led to the hypothesis that ORMDL3 might play a role in the regulation of the immune system, somehow facilitating the development of inflammatory diseases. However, the functions of the ORMDL proteins are just about to reveal and subject in this study; to date their involvement in calcium homeostasis, ER stress and UPR response and *de novo* sphingolipid biosynthesis regulation is described. Several cellular subsets of the immune system have been investigated, such as eosinophiles, mast cells and T-lymphocytes and showed impairment of their activational process. In this thesis we focused on macrophages, as they display key players in the innate immunity and play a central role in early pathogen recognition and cross-talk with other cellular subsets during inflammation. According to the ORMDLs function as *de novo* sphingolipid regulators, the macrophage activation is an extremely interesting process of investigation, since its proper induction depends on *de novo* sphingolipids. This thesis provides a better understanding of the regulatory role of ORMDLs in physiological macrophage activation, the impact of sphingolipids in this process and important consequences. In addition, we evaluate if the activation of human monocytes shed light on possible relation to its pathophysiology.

Abbreviations

AP-1	Activator protein 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATG	Autophagy-related gene
BAL	Bronchioalveolar lavage
BiP/ Grp78	Glucose-regulated protein 78
BMDM	Bone marrow-derived macrophages
C/EBP	CCAAT/enhancer- binding protein
C1P	Ceramide-1-phosphate
CCL2	Chemokine (C-C motif) ligand 2
CD14	Cluster of differentiation 14
CERK	Ceramide kinase
CerS	(Dihydro)-ceramide synthase
CERT	Ceramide transfer protein
CNS	Central nervous system
CoA	Coenzyme A
CREB	cAMP response element binding protein
DAG	Diacylglycerol
DES	Dihydroceramide desaturase
DHcer	Dihydroceramide
eIF2a	Eukaryotic translation initiation factor alpha
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GM-CSF	Granulocyte macrophage colony stimulating factor
GSDMB	Gasdermin B
GWAS	Genome wide-association studies
HEK	Human embryonic kidney
HSAN-I	Hereditary sensory and autonomic neuropathy type I
IBD	Inflammatory bowel disease
IKZF3	Ikaros zinc finger protein 3
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-13	Interleukin 13

INF-y	Interferon-y
$InsP_3R$	Inositol- 1,4,5-trisphosphate receptor
IRE1	Inositol-requiring protein 1
IRF-3	Interferon regulatory transcription factor 3
IRS-2	Insulin receptor substrate 2
KDHR	3-KetoDiHydrosphingosine reductase
LBP	LPS binding protein
LC	Langhans cells
LC3	Light chain 3
LPS	Lipopolysaccharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
M-CSF	Macrophage-colony stimulation factor
MD-2	Myeloid differentiation factor 2
MHC II	Major histocompatibility complex class II
MMR/CD206	Macrophage mannose receptor
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation factor 88
NFAT	Nuclear factor of activated T-cells
NfκB	Nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells
NK	Natural killer
NLR	NOD-like receptos
NO	Nitric oxide
ORMDL	Orosomucoid-like
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PERK	Protein kinase-like ER kinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PMCA	Plasma membrane Ca^{2+}-ATPase
PRR	Pattern recognition receptor
ROC	Receptor-operated channel
RYR	Ryanodine receptor
S1P	Sphingosine-1-phosphate
SERCA	Sarco-endoplasmic reticulum ATPase
SK	Sphingosine kinase

SM	Sphingomyelin
SMS	Sphingomyelin synthase
SNP	Single nucleotide polymorphisms
SOC	Store-operated channel
SOCE	Store-operated calcium entry
SPL	Sphingosine-1-phosphate lyase
SPT	Serine palmitoyltransferase
SR	Sarcoplasmic reticulum
STAT6	Signal transducer and activator of transcription 6
Th2	T-helper 2 cells
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor inducing interferon-b
TSS	Transcription start sequence
TYK2	Tyrosine kinase 2
ULK1/2	UNC-51-like kinase1/2
UPR	Unfolded protein response
VOC	Voltage-operated channel
γc	γ-chain

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I. INTRODUCTION

1. ORMDL family

The evolutionary conserved gene family encoding for orosomucoid-like (ORMDL) proteins was first reported by Hjemlqvist and colleagues. The *ORMDL1* gene was identified in the same genomic region as the initially characterized retinitis pigmentosa locus RP26 at 2q31-q33 [1]. Full-length ORMDL1 as template in a nucleotide database search revealed that this family comprises two additional members in humans, *ORMDL2* (12q13.2) and *ORMDL3* (17q21.1). Although the human ORMDL homologs mapped to different chromosomes, they show a conserved genomic structure of three coding exons as well as exon-intron boundaries. All three genes are ubiquitously expressed in adult and fetal tissues with a lower expression in heart, brain and skeletal muscle. Interestingly, the number of genes varies in invertebrates: *S. cerevisae* and *A. thalia* showed two copies each, while *C. intestinalis, D. melongaster, S. monacensis* only displayed one single copy.

The human ORMDL proteins consist of 153 amino acids with an estimated molecular mass of 17.4 kDa and share around 80% of sequence identity. Pairwise comparison between human and mouse orthologs showed more than 95% of identity. It can be visualized in a phylogenetic tree that each human ORMDL protein has a conserved counterpart in vertebrates, separating them from the other two branches of plant and yeast (Fig.1).

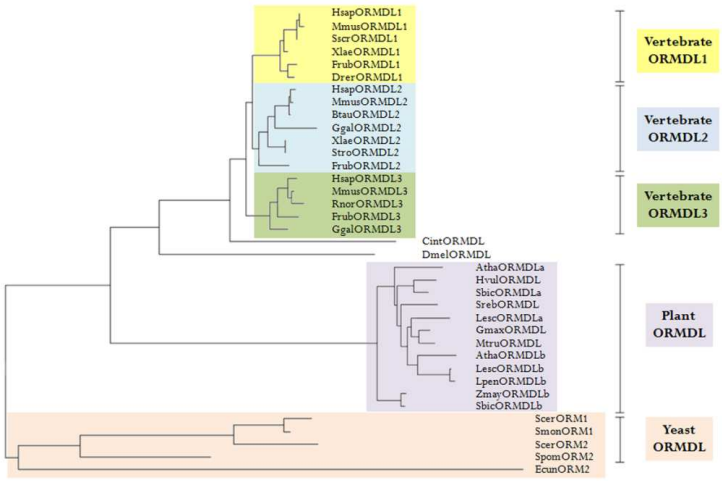

Figure 1. Phylogenetic tree of ORMDL aminoacid sequences. Vertebrate ORMDL1 are highlighted in yellow; vertebrate ORMDL2 in blue, vertebrate ORMDL3 in green, plant ORMDL in purple, and yeast ORMDL in orange. Species abbreviations are as follows: Hsap, human; Mmus, mouse; Rnor, rat; Sscr, pig; Btau, cow; Ggal, chicken; Xlae, *Xenopus laevis*; Stro, *Silurana tropicalis*; Frub, *Takifugu rubripes* (pufferfish); Drer, *Danio rerio* (zebrafish); Cint, *Ciona intestinalis*; Dmel, *Drosophila melanogaster*; Atha, *Arabidopsis thaliana*; Hvul, *Hordeum vulgare* (barley); Sbic, *Sorghum vulgare*; Sreb, *Stevia rebaudiana*; Lesc, *Lycopersicon esculentum* (tomato); Gmax, *Glycine max* (soybean); Mtru, *Medicago truncatula*; Lpen, *Lycopersicon pennellii*; Zmay, *Zea mays* (maize); Scer, *Saccharomyces cerevisiae*; Smon, *Saccharomyces monacensis*; Spom, *Schizosaccharomyces pombe*; Ecun, *Encephalitozoon cuniculi*. Adapted from Hjelmiqvist *et al*., 2002 [1].

As mentioned above, there are only two orthologs described in yeast, namely Orm1 and Orm2 with a sequence identity of about 35% compared to human orthologs. These proteins exhibit longer amino- and carboxy-terminal tails than human ORMDLs. Single and double knockout mutants were viable and showed no morphological changes [1].

1.1 Protein structure and location

Subcellular localization of the ORMDL proteins in the Endoplasmic Reticulum (ER) has been shown by immunofluorescence studies using heterologous expression systems and specific antibodies against the

endogenous ORMDLs in various cell types [1–6].

ORMDLs are transmembrane proteins, however there is controversy about the number of segments crossing the ER membrane, since protein sequence alignment studies led to the prediction between two to four putative transmembrane domains [1,2,7]. Based on fluorescence protease protection assays, our laboratory proposed a structural model of two transmembrane domains with a luminal loop inside the ER lumen, in which the amino- and carboxy-terminal ends were facing the cytosol [3]. The human protein structure of ORMDL3 is represented in Figure 2 by its amino acid sequence.

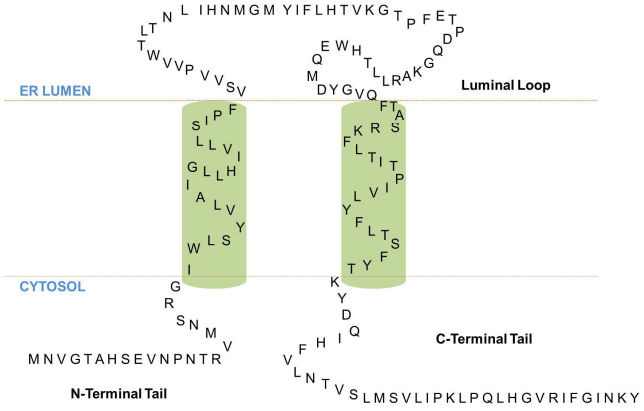

Figure 2. Structure of human ORMDL3. Aminoacid sequence of human ORMDL3 displays two transmemebrane segments with a luminal loop, whereas C- and N- terminal tails face the cytosol. Image obtained from Carreras, 2014 [8].

1.2 Genome wide association studies

Genome wide association studies (GWAS) provide a powerful tool to analyze DNA sequence variation across the human genome in order to identify genetic risk factors for complex diseases. Therefore the genome of a healthy control population is sequenced and compared to one of a

disease state and single nucleotide polymorphisms (SNPs) are analyzed. SNPs are single base pair changes associated with a disease if one type of the variant or one allele is occurring with a higher frequency in people with the disease [9].

The unbiased GWAS approach has reconfirmed existing data on candidate gene studies with an increased cohort size and is an opportunity to discover novel target genes. But it is a challenge to discard non-causal SNPs that are simply in linkage disequilibrium with disease-causing genes. Therefore, functional studies are inevitable to confirm biological relevance for those SNPs.

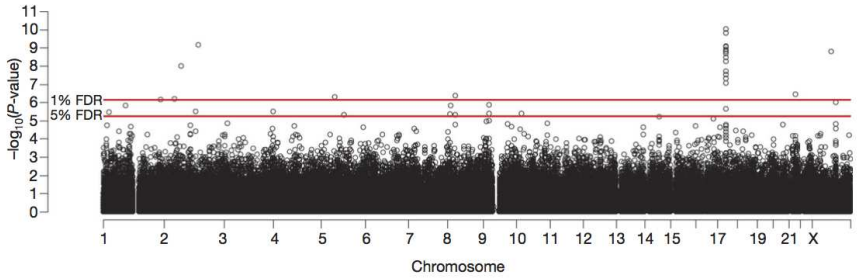

Figure 3. Genome-wide association of SNPs with asthma. 317,447 SNPs have been linked to asthma in this study with 994 asthmatic children and 1,243 non-asthmatic children. Depicted on the x axis is the position in the genome divided by chromosomes; the y axis displays strength of association. Every black circle demonstrates the result for each individual marker, whereas the red horizontal lines define the genome-wide thresholds for 1% and 5% false discovery rates (FDR). Chromosome 17q21 shows numerous markers of association to asthma above the 1% FDR threshold in the region of maximum association. Image reproduced from Mofatt *et al*. 2007[10].

Accordingly, GWAS were used to identify genes that might be involved in asthma pathogenesis [11]. Multiple SNPs were elucidated on chromosome 17q21 that linked ORMDL3 to the risk of developing childhood asthma [10] (Fig.3). In this study the SNP with the strongest evidence of association was rs7216389. It is located within the first intron of the neighboring

gasdermin B (GSDMB) gene. Part of this non-coding region shows high homology to the pro-inflammatory transcription factor CCAAT-enhancer-binding protein beta (C/EBPß). Genetic variation at this SNP (T allele) was shown to increase *ORMDL3* transcript levels, thereby determining the susceptibility to childhood asthma [10]. The pooled odds ratio of the T allele was 1.37 as obtained in a meta-analysis combining all 11 published association studies of rs7216389 polymorphism with asthma [10,12–21].

Several studies have consistently replicated *ORMDL3* as a candidate gene for asthma susceptibility in ethnically distinct populations in GWAS and non-GWAS genetic association studies [10,13–15,20,22–26].

Moreover, the 17q21 asthma susceptibility locus and ORMDL3 has been linked to other diseases, such as Crohn's disease [27–29], primary biliary cirrhosis [30–32], diabetes type 1 [33], ulcerative colitis [2], glioma [34], white blood cell count [35], rheumatoid arthritis [36,37], allergic rhinitis [38], cervical cancer [39] and IL-17 production [40], suggesting the implication of ORMDL3 in dysregulating the immune system.

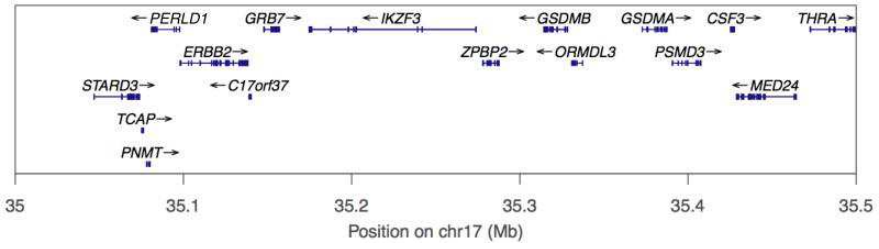

Figure 4. Schematic of chromosome 17. The region between 35.0 and 35.5 Mb on chromosome 17 is displayed with the *ORMDL3* surrounding genes. Vertical lines delineate asthma-associated SNPs. Image reproduced from Granell *et al.* 2013[41].

The asthma susceptibility locus is located between 35.0 and 35.5 Mb on chromosome 17 and contains at least 15 genes. However, the expression of only three of these genes have been associated to asthma-related SNPs,

which are the Ikaros zinc finger protein 3 (IKZF3), playing a role in the regulation of lymphocyte development; GSDMB, involved in epithelial cell barrier function and ORMDL3 [42] (Fig.4).

GSDMB and ORMDL3 have been most intensively studied, because their transcript levels seem inter-connected in a cis-regulation [21]. Moreover, rhinovirus infection was shown to affect the genotype-mediated expression of both genes, which is known to induce asthma exacerbations [43]. The allele-specific expression of ORMDL3 [10] was proposed to be the functional mechanism for the predisposition to asthma, but the subsequent discovery that gene regulation also occurs for other transcripts in the same region pointed towards a more complex regulatory mechanism.

1.3 Regulation of mammalian ORMDL3 expression

The *ORMDL3* promoter is fully unmethylated independent from the genotype, thereby linking gene transcription directly to genetic effects and allele expression [44]. The putative promoter region for the major *ORMDL3* isoform showed high promoter activity in all cell lines tested with no allelic effect. However, allelic variation in expression has been postulated to arise from the interaction between several genetic polymorphisms and epigenetic factors. A genomic region overlapping with the *ZPBP2* gene at least 31 kb away from the *ORMDL3* gene exerts in vitro promoter and enhancer activity as shown due to its nucleosome occupancy and DNA methylation [42].

The ORMDL3 promoter was first claimed to be located at position -84/+58 in reference to the transcription start sequence (TSS). Mutation of this region revealed that ORMDL3 expression was under the control of Ets-1, p300 and CREB [45] (Fig.5).

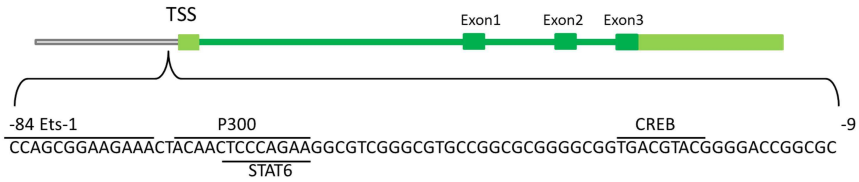

Figure 5. Minimum promoter region of human ORMDL3. Schematic representation of the *ORMDL3* gene with its three exons. Sequence and putative transcription factor binding sites of the minimal promoter of *ORMDL3* are underlined. Image is based on Lin *et al*. 2012 and Qui *et al*. 2013 [45].

Interestingly, intranasal administration of interleukin 4 (IL-4) and interleukin 13 (IL-13) in mice induced signal transducer and activator of transcription 6 (STAT6) activity, as well as *ORMDL3* expression in lung epithelial cells [4]. Based on the previously published ORMDL3 promoter sequence [45], no STAT6 binding site was identified. Suggesting that STAT6 is indirectly regulating *ORMDL3* expression via a STAT6-dependent mediator in lung epithelial cells [4]. In the same study the implication of STAT6 in ORMDL3 induction was confirmed using STAT6 KO mice.

Another study evaluated the occurrence of a STAT6 binding motif in the proximal murine promoter of ORMDL3 and found a possible binding site in human with a 76% homology compared to its consensus sequence, which showed a strongly decreased promoter activity after point mutations [46].

The subsequent characterization of the ORMDL3 promoter using luciferase assays narrowed the minimum promoter sequence to the position -64/-56 relative to the TSS. The STAT6 binding motif located in this region was shown to interact with P300 [47].

ORMDL3 expression also seems to be regulated by phosphorylation of CREB transcription factor via the cAMP-dependent protein kinase A (PKA) allowing CREB binding to the proximal human and mouse promoter, thereby initiating transcription of ORMDL3 [46,48].

1.4 ORMDL function

The ORMDL protein family was first postulated to participate in correct protein folding and trafficking in the ER. Moreover, these proteins seem to be involved in the Unfolded Protein Response (UPR) due to their location and response to ER stress inducers like DTT and Tunicamycin [1]. Nevertheless, the specific function remained unknown until a genome-wide association study (GWAS) linked ORMDL3 expression levels to the susceptibility of childhood asthma [10]. This finding raised the interest of the scientific community and led to further studies of the ORMDL family. Besides, in the following years ORMDL3 was associated to other pro-inflammatory diseases, such as asthma [10,13,22,26,49–51], inflammatory bowel disease (IBD) [2,27,28], diabetes type 1 [33] and rheumatoid arthritis [36,37].

Until now this protein family has been shown to participate in three mayor cellular processes: ER stress and UPR, intracellular calcium signaling and the *de novo* sphingolipid synthesis. In order to have a better understanding of the function of this family of proteins, a general overview of every process will be provided before the description of the role in each of them.

1.1.1 ER stress and UPR

The endoplasmic reticulum is a cellular organelle involved in secretory and membrane protein folding, lipid biosynthesis and calcium storage [52,53]. Therefore, disturbance of the ER homeostasis by exceeding or impairing its folding capacity leads to an abnormal protein accumulation, generally

referred to as ER stress. ER stress can also be provoked by a variety of pathophysiological conditions such as nutritional deficiency, viral infections or mutations [54,55]. In these ER stress situations an important cellular response in order to restore the ER homeostasis is the unfolded protein response (UPR). It combines at least three components: (1) the translational attenuation of protein synthesis to prevent further loading of proteins into the ER, (2) transcriptional induction of chaperones, enzymes and structural components of the ER to enhance protein folding efficiency and (3) the induction of ER-associated degradation (ERAD) to eliminate misfolded proteins [56–58]. If the overload of unfolded proteins is sustained and cannot be resolved cells undergo apoptosis.

The UPR is a tightly regulated signaling cascade controlled by three main stress sensors: inositol-requiring protein 1 (IRE1), double- stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) [59] (Fig.6).

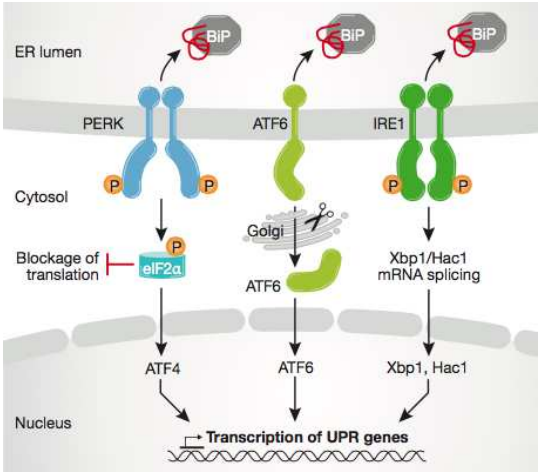

Figure 6. Branches of the UPR pathway. The mammalian Unfolded Protein Response (UPR) consists of the three ER stress sensor proteins PERK, ATF6 and IRE1. Their specific signaling cascades are displayed, ultimately leading to the transcription of UPR genes. Modified image from Douglas *et al*. 2009 [60].

BiP regulation

All three UPR sensors get activated via dissociation of the ER-resident chaperone glucose-regulated protein of 78 kDa (GRP78), also known as BiP, which is bound to each of their ER luminal domain in resting conditions [61,62]. This binding maintains IRE1 and PERK in a monomeric state. Misfolded protein accumulation triggers dissociation from BiP allowing their oligomerization and phosphorylation [61,63]. The ER stress activation of the different UPR signaling pathways is depicted in Fig. 3.

IRE1

Upon ER stress IRE1 gets trans-autophosphorylated to promote its endoribonuclease activity at the cytosolic tail. Once activated it regulates the non-conventional mRNA splicing of the transcription factors Hac1 and XBP1 to mediate transcriptional induction of chaperones and quality control factors [64–67].

PERK

ER stress induces PERK-dependent phosphorylation of the eukaryotic translation initiation factor alpha (eIF2a), which leads to global attenuation of protein synthesis [68,69] and selective translation of the activating transcription factor 4 (ATF4). ATF4-mediated induction of its downstream target gene *CHOP (GADD153)* insures supply of amino acids and protection against oxidative stress [70–72].

ATF6

ATF6 dissociates from the ER chaperone BiP in response to ER stress and is thereafter transported to the Golgi. Subsequent proteolytic cleavage by site-1 and site-2 proteases in the Golgi releases a cytosolic fragment

(p50ATF6) [73–75]. This active form of ATF6 translocates to the nucleus to encode genes for the ERAD machinery and ER chaperones [73,76].

1.1.1.1 Involvement of ORMDLs in UPR

ORMDLs were postulated to be involved in UPR signaling pathways soon after their discovery. Hjelmqvist and colleagues in 2002 found that double-knockout yeast mutants showed growth impairment after treatment with DTT and Tunicamycin, which are known inducers of unfolded protein accumulation in the ER lumen. This result has been reproduced by several other labs [77–79].

However, the involvement of human ORMDLs in ER stress and UPR has been of controversial matter. The first study in human embryonic kidney cells 293 (HEK293) was published by our laboratory linking ORMDL3 overexpression to an increase in eIF2a phosphorylation, whereas XBP-1 mRNA splicing, a process downstream IRE1 activation, was not altered. Concluding that higher levels of ORMDL3 induce UPR mainly via the PERK signaling pathway [3].

Another study showed a decrease in basal and ER stress induced UPR after ORMDL3 overexpression as assessed by luciferase activity monitoring the UPRE transcription activation following XBP1 splicing downstream of the IRE1/ATF6 pathway [2].

Induction of the ATF6 signaling pathway has been observed in lung epithelial cells (A549) after ORMDL3 overexpression and in bone marrow-derived macrophages (BMDM) from transgenic ORMDL3 knock-in mice by ATF6 nuclear translocation studies [4,6]. Whereas the UPR branches like IRE1 (assessed by Xbp-1 mRNA splicing) and PERK (assessed by eiF2a phosphorylation) showed no alterations in A549 cells[4].

Finally, a study performing siRNA knockdown of ORMDL3 in airway epithelial cells (1HAE) and A549 cells reported no differences in any of the three UPR branches as assessed by previously described methods [80].

Considering these evidences ORMDL proteins do not have a clear role in the UPR pathway. However, since altering ORMDL3 expression level activates UPR branches in different models, it seems clear that their activity is essential for ER homeostasis.

1.1.2 Calcium homeostasis

Calcium (Ca^{2+}) is an important divalent cation acting as a universal second messenger for intracellular cell signaling and participates in numerous biological processes: from muscle contraction to neuronal activity, and from gene expression to cell proliferation and death (reviewed in [81]). Therefore, intracellular Ca^{2+} concentration needs to be tightly regulated in order to ensure proper coordination and cell functioning among the versatility of processes.

The cellular Ca^{2+} signaling network is established by a gradient between the intracellular (around 100 nM) and extracellular (mM) Ca^{2+} concentration allowing the cell to stimulate other Ca^{2+}-sensitive processes depending on speed, amplitude and spatio-temporal pattern of the Ca^{2+} influx [82,83]. Several stimuli trigger the rise in the fairly low cytosolic Ca^{2+} concentration under resting conditions. The so-called ON mechanisms summarize the increase of cytosolic Ca^{2+} that can originate from either entry of external Ca^{2+} or its release from internal stores. The entry of external Ca^{2+} is performed by differentially activated Ca^{2+} channels located on the plasma membrane, such as voltage-operated channels (VOCs), receptor-operated channels (ROCs) and store-operated channels (SOCs). The release from internal stores is mainly controlled by two

channel families located in the ER, or in the so-called sarcoplasmic reticulum (SR) in muscle cells, the inositol- 1,4,5-trisphosphate receptors ($InsP_3R$) and ryanodine receptors (RYR) [84,85]. On the other hand, the OFF mechanisms combine strategies for fast removal of free Ca^{2+} from the cytoplasm carried out by several pumps and exchangers [52,86]. The transport of Ca^{2+} out of the cell is performed by plasma membrane Ca^{2+}-ATPase (PMCA) pumps and Na^{+}/ Ca^{2+} exchangers both located at the plasma membrane, whereas the sarco-endoplasmic reticulum ATPase (SERCA) pump returns Ca^{2+} to the internal stores. Additionally, the mitochondrion has an enormous Ca^{2+} capacity, which is rapidly accumulated during the development of Ca^{2+} signals and then slowly released during the recovery phase [87–89] (Fig.7).

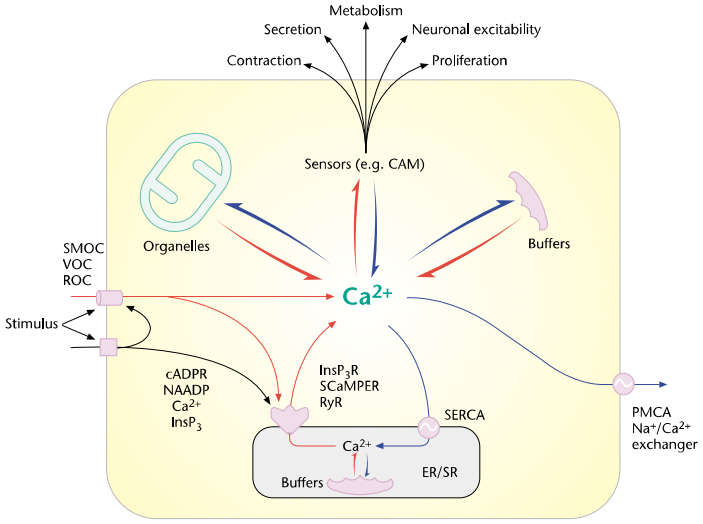

Figure 7. Basic mechanisms of Ca^{2+} signaling. External stimuli activate the Ca^{2+} ON reactions (red arrows) responsible for increasing cytosolic Ca^{2+} through plasma membrane channels or from internal calcium stores. Ca^{2+} OFF reactions remove Ca^{2+} from the cytoplasm to return to resting levels. Modified image from Bootman *et al*. 2006 [90].

1.1.2.1 Involvement of ORMDLs in UPR

Our laboratory described the first mechanistic link between ORMDL3 and calcium homeostasis using a heterologous expression system in HEK cells [3]. Overexpression of ORMDL3 not only showed a higher basal cytosolic Ca^{2+} concentration, but also a reduced amount of Ca^{2+} stored in the ER. Slower kinetics of cytosolic Ca^{2+} reuptake into the ER after stimulation is indicative for impaired SERCA activity [91–94]. ORMDL3 was shown to directly interact with and inhibit SERCA pump, thereby altering cellular calcium homeostasis [3]. Moreover, SERCA2b transcriptional induction has been shown to depend on ORMDL3 expression levels [4,6].

A functional link between ORMDL3 levels and calcium homeostasis has been provided in cells of the immune system: T-lymphocyte activation is dependent on Ca^{2+} signals following antigen binding to the T-cell receptor. Elevation of cytosolic Ca^{2+} due to ER depletion is augmented by a process called store-operated calcium entry (SOCE) [95] promoting the translocation of nuclear factor of activated T-cells (NFAT) and subsequent transcription of the activation marker interleukin 2 (IL-2) [96]. Overexpression of ORMDL3 has been shown to reduce SOCE, thereby negatively affecting T-cell activation as measured by NFAT translocation to the nucleus and IL-2 production. Moreover, a reduced mitochondrial Ca^{2+} uptake was shown at high levels of ORMDL3 [97]. Placing the ORMDLs at an important junction point of calcium homeostasis between ER and mitochondrion, as both organelles are functionally interconnected at the mitochondrial-associated membranes (MAMs) [98,99].

The importance of Ca^{2+} signaling has also been demonstrated in eosinophils, which showed decreased cytosolic Ca^{2+} levels after ORMDL3 knockout impairing the migration and degranulation of these cells [5].

2. Sphingolipid Synthesis

Regulation of sphingolipid synthesis is one of the functions of ORMDL proteins. Since this thesis is mainly focused on ORMDL implication therein, we present the detailed description in a separate chapter.

2.1 Structure and function

Sphingolipids belong to a class of lipids composed of a sphingoid base backbone that is made of a 18 carbon atom-containing amino alcohol. In mammals sphingosine is the predominant sphingoid base, which represents the basic structure of ceramide that has a long chain fatty acid attached to the amino group. Ceramide can be further converted to more complex sphingolipids by attachment of neutral, charged, phosphorylated or glycosylated moieties (Fig. 8). Sphingolipds are structural components of the membrane (10-20% of membrane lipids) due to their amphipathic character as they contain polar and non-polar regions. Moreover, sphingolipids can act as signaling molecules. The structural diversity of these molecules explain their widespread involvement in cellular metabolism, such as signal transduction, cell growth, differentiation, adhesion, migration, senescence, inflammation and apoptosis [100–104]. In this context, sphingosine, ceramide, sphingosine-1-phosphate (S1P), and ceramide-1-phosphate (C1P) are the main sphingolipid derivates involved in cellular signaling events. Whereas sphingomyelin, which contains a phosphorylcholine attached to ceramide is the most abundant mammalian sphingolipid and functions as a structural membrane component.

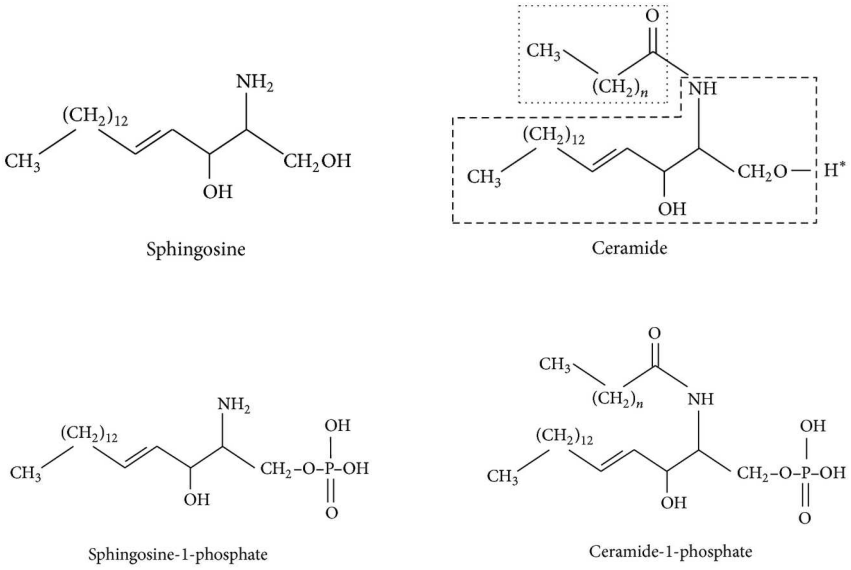

Figure 8. Chemical structure of important signaling sphingolipids.

2.2 The metabolism of sphingolipids

The metabolism of sphingolipids has been studied extensively and represents a tightly coordinated interplay of biochemical pathways and enzymatic reactions [105]. Therein, ceramide plays a central role in both sphingolipid synthesis and degradation and has been previously described as a metabolic hub [106]. Overall, intracellular sphingolipids originate from extracellular uptake, degradation from more complex sphingolipids and *de novo* sphingolipid synthesis (Fig. 9).

2.2.1 De novo synthesis

The initial reaction of the *de novo* sphingolipid synthesis is the condensation of cytosolic L-serine and palmitoyl-Coenzyme A (CoA). This reaction is catalyzed by the rate-limiting enzyme serine palmitoyltransferase (SPT) to produce 3-ketodihydrosphingosine (mostly referred to as 3-ketosphinganine) [107]. Followed by the rapid enzymatic

reduction of 3-ketodihydrosphingosine at its ketone group to convert it to a hydroxyl group to form dihydrosphingosine (or sphinganine) catalyzed by the 3-KetoDiHydrosphingosine Reductase (KDHR) in a NADPH dependent manner. The third reaction is carried out by (dihydro)-ceramide synthase (CerS), which acylates sphinganine to dihydroceramide by addition of a fatty acyl-CoA. This reaction is well-adjusted, as in mammals six different CerS have been described, each encoded by a distinct gene [108,109]. Interestingly, each CerS has a primary acyl-CoA preference, thereby producing a specific dihydroceramide profile. CerS1 utilizes mainly stearoyl-CoA and generates C18-ceramide species [110]; CerS2 is producing long chain ceramide species by using C20-C26 as substrates [111]; CerS3 utilizes middle to long chain acyl-CoA substrates and is mainly expressed in testis and epidermis [112,113]; whereas CerS5 and CerS6 prefer palmitoyl-CoA as a substrate to generate C16-ceramide species [114,115]. To date it is still unknown how sensitive bioactive modulators of the ceramide metabolism are to changes in acyl chain length and how CerS activity is regulated to potentially influence cellular fate. The expression of the different CerS varies between tissues and the *de novo* production of dihydroceramide can be induced by various stress stimuli. Moreover, the binding of sphingosine-1-phosphat (S1P) –a sphingolipid characteristic of ceramide breakdown- to its binding site in exclusively CerS2, leads to CerS2 inhibition, suggesting a negative regulation of CerS activity [111]. The last reaction of the *de novo* sphingolipid synthesis is carried out by the enzyme dihydroceramide desaturase (DES) with the introduction of a hydroxyl group and subsequent dehydration using NADPH to form a double bond [116–118]. The product of the desaturation reaction with a double bond between C4-C5 is called ceramide.

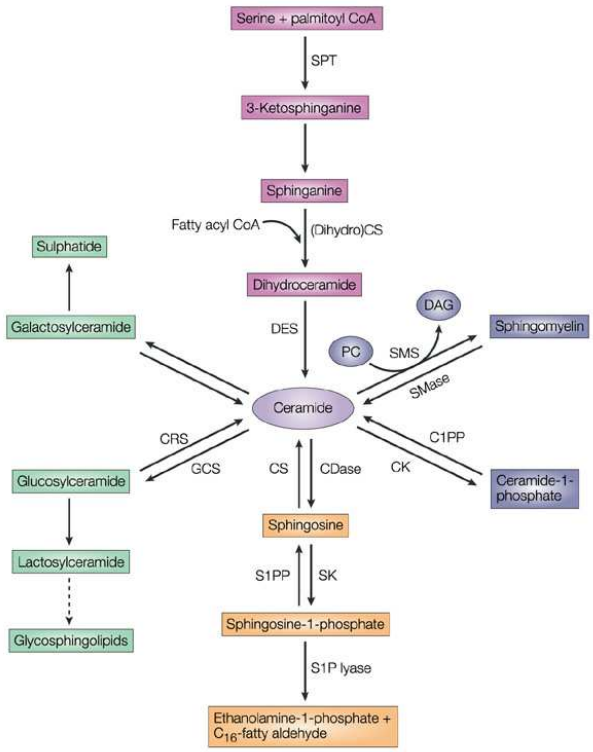

Figure 9. Major pathways of sphingolipid metabolism. This diagram shows different pathways of ceramide production and degradation. The *de novo* sphingolipid synthesis is displayed in pink. Ceramide can also be formed from the hydrolysis of cerebrosides (green) or sphingomyelin (blue) and metabolized by ceramidases (orange). Image obtained from Ogretmen *et al*., 2004 [119].

The entire process of *de novo* sphingolipid synthesis is taking place at the cytosolic leaflet of the ER. All four enzymes involved are embedded in the ER membrane with their catalytic sites facing the cytosol. In order to function as a signaling molecule and to be further processed to more complex sphingolipids, membrane-bound ceramide with a very low solubility in aqueous environments needs to be transported to the Golgi.

2.2.2 Transport from ER to Golgi

The ceramide transport from the ER to Golgi can be mediated by a vesicular and a non-vesicular mechanism. The non-vesicular transport is performed by the ceramide transfer protein (CERT), which comprises donor membrane (ER) and acceptor membrane (Golgi) recognition domains and a hydrophobic pocket for direct ceramide binding. CERT shows preference for ceramides with an acyl chain length less than C22 [120,121]. The vesicular transport is much less characterized, however it might be the main pathway of ceramide transport to the cis-Golgi [122]. The transported ceramides are then further processed to complex shphingolipids like sphingomyelin, galactosylsphingolipids glucosylsphingolipids that differ from the residue attached to the C1-headgroup of ceramide [123].

2.2.3 Sphingomyelin synthesis

The most abundant mammalian complex sphingolipid is sphingomyelin generated via the transfer of a phosphocholine headgroup from phosphatidylcholine to ceramide by sphingomyelin synthase (SMS) resulting in diacylglycerol (DAG) and sphingomyelin (SM). Sphingomyelin plays an essential role in cell viability, because inhibition of its production by CERT mutation or defects in *de novo* sphingolipid synthesis leads to cell death [124]. Interestingly, ceramide and DAG show opposite effects on proliferation and survival, suggesting that SMS has a regulatory function therein. To reverse its accumulation sphingomyelin can be hydrolyzed to ceramide via sphingomyelinases.

2.2.4 Phosphorylation of ceramide

Ceramide is mainly used to form more complex sphingolipids, but its phosphorylation is another process that occurs in the trans-Golgi by

ceramide kinase (CERK). CERK shows preference for ceramides with an acyl chain length of more than C12 and its activity is enhanced by the presence of calcium and magnesium to form ceramide-1-phosphate (C1P) [125]. C1P enrichment coming from specific ceramide precursors in several cell types or tissues is partially influenced by the selective CERT transport to the Golgi [126]. Addition of C1P to BMDM after M-CSF removal resulted in a decreased apoptosis induction, suggesting an important role in cell survival [127]. C1P can also be converted back to ceramide by the action of phosphatases located in the plasma membrane [128].

2.2.5 Degradation

Catabolizing sphingolipids is an extremely important mechanism in order to circumvent the overload of lipids in cells or tissues, especially because their hydrophobicity impedes excretion. All complex sphingolipids follow the stepwise degradation to ceramide, sphingosine and finally sphingosine-1-phosphate (S1P). The enzymatic deacylation of ceramide to produce sphingosine is performed by tissue-specific ceramidases, classified by their pH optima. The subsequent phosphorylation to form S1P is carried out by two distinct Sphingosine Kinases (SK1; SK2). Translocation of SK1 from the cytosol to the plasma membrane upon stimulation enhanced its activity and led to extracellular release of the signaling molecule S1P [129], thereby favoring cellular proliferation and survival [130,131]. SK2 is mainly localized in the nucleus with wider substrate specificity than SK1. In contrast to SK1, it was shown to induce apoptosis upon overexpression [132], since the generated S1P in the nucleus distal from the membrane is less likely to be exported.

The final step in sphingolipid degradation is the conversion of S1P to hexadecenal and phosphoethanolamine by Sphingosine-1-Phosphate Lyase (SPL), an enzyme exclusively localized to the ER [133].

2.3 Serine Palmitoyl Transferase

2.3.1 Structure of SPT

SPT, the enzyme initializing *de novo* sphingolipid synthesis, was described as a heterodimer bound to the outer membrane of the endoplasmic reticulum and its activity is dependent on the cofactor pyridoxal 5′-phosphate (PLP) [134]. Each SPT subunit is composed of several putative transmembrane domains with the N-terminus facing the ER lumen and the C-terminus facing the cytosol, as does the catalytic site [135]. Three genes encode SPT, namely SPTLC1, SPTLC2 and the more recently identified SPTLC3 [107,136]. The subunits of SPTLC1 (55 kDa) and SPTLC2 (65 kDa) show 20% similarity and are highly conserved among species, whereas SPTLC2 and SPTLC3 (63 kDa) share more than 80% of similarity [137]. The active heterodimeric form of SPT is composed of the subunit SPTLC1 and either SPTLC2 or SPTLC3, of which only the latter two contain a PLP binding site and are therefore being considered as the catalytic subunits [134,138]. They differ in their acyl-CoA recognition affinities: SPTLC2 metabolizes predominantly palmitoyl-CoA; whereas SPTLC3 utilizes shorter acyl-CoAs, like lauroyl- and myristol-CoA [139]. The requirement of SPTLC1 apart from contributing to the catalytic activity was proposed to lay in the stabilization of the complex within the ER [140]. Interestingly, it was also proposed that the native SPT complex is composed of all three subunits, as a large protein complex of 460 kDa was detected by immunoprecipitation, native gel analysis, cross-linking and size exclusion chromatography studies [141].

Additionally, two so-called small subunits of the human SPT complex (ssSPTa, ssSPTb) were described to be required for maximal enzyme activity. Both isoforms activate the SPT complex (either dimerized with SPTLC2 or SPTLC3) by direct interaction with the SPTLC1 subunit,

further determining acyl-CoA preference [142].

2.3.2 Expression of SPT

SPT is ubiquitously expressed with a tissue-specific pattern of the different subunits, thereby favoring a SPT complex formation with the predominant catalytic subunit: SPTLC1 is relatively low expressed throughout the different tissues; it only shows higher expression in pancreas, placenta and lung. SPTLC2 is high in lung, bone marrow, spleen, stomach and peripheral blood cells. SPTLC3 is high in placenta, skin, adrenal gland, testis, uterus, salivary, prostate and kidney. Interestingly, SPTLC3 is absent in peripheral blood cells and bone marrow [137]. SPT plays an essential role in embryonic development, because homozygous SPTLC1 and SPTLC2 knock-out mice die during embryogenesis [138].

2.3.3 Regulation of SPT

SPT activity needs to be well adjusted in order to maintain cellular sphingolipid homeostasis. In this respect, there are several inducers of SPT activity, such as UVA, Endotoxins and Tetrahydrocannabinol[143–146]. SPT is not only regulated at the transcriptional level, but is also blocked by specific inhibitors. The most potent and widely used natural inhibitor of SPT is myriocin (Myr, Ki=0.28nM) acting on the catalytic site, among others such as L-penicillamine, sphingofungin B and cycloserine [147–149] (Fig. 10). Nevertheless, the exact mechanisms of inhibition are not very well understood. Moreover, ORMDL proteins have been recently described as endogenous negative regulators of SPT activity.

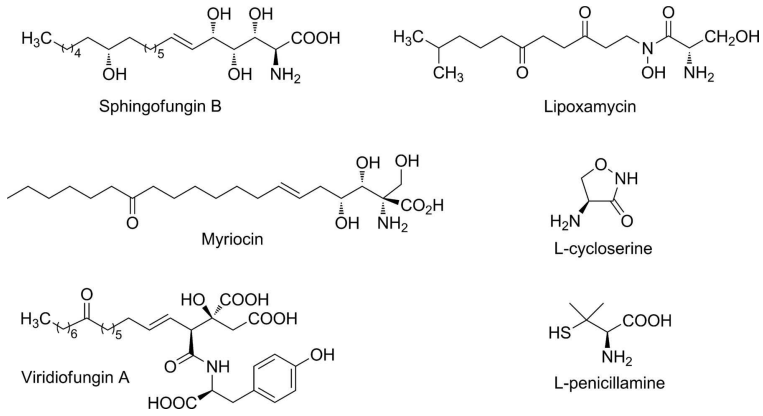

Figure 10. Inhibitors of SPT. Several natural product inhibitors of the first rate-limiting enzyme of *de novo* sphingolipid synthesis. Image obtained from Lowther *et al*. 2012 [150].

2.4 Involvement of ORMDLs

The first experiments claiming that ORMDL proteins are involved in sphingolipid synthesis were performed in yeast. The two main observations confirming this hypothesis were: 1) double-KO of Orms caused higher levels of sphingolipids and 2) there was a direct interaction between Orms and both yeast SPT subunits [77,78].

Further studies revealed, that the SPT regulation by Orms is dependent on cellular sphingolipid levels. Low levels lead to the phosphorylation of both Orm orthologs at their N-terminal tails. Orms get phosphorylated by two different kinases: YPK, downstream of TORC2 [151] and Npr1 downstream of TORC1 [152]. Multiple phosphorylation sites provoke the gradual dissociation from SPT, thereby releasing its activity to cover the demand of sphingolipids [77]. On the other hand side, if sphingolipid levels are high, two different phosphatases can dephosphorylate the Orms: Tap42 phosphatase complex downstream TORC1 [79] and Cdc45-PP2A, which is activated under the heat stress response [153]. Taken together, these findings place Orms as negative regulators of SPT at a critical step of the

de novo sphingolipid synthesis. Where they act in support of sphingolipid homeostasis via a phosphorylation-mediated feedback loop (Fig. 11). In this respect, it is important to state that the regulatory region of the N-terminus with multiple phosphorylation sites described in yeast is absent in the mammalian ORMDL isoforms [1,77]

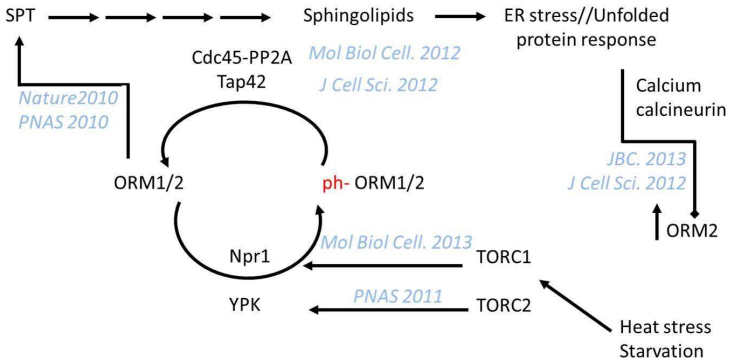

Figure 11. Orm1 and Orm2 participate in sphingolipid homeostasis. Orm1 and Orm2 inhibit SPT activity, thereby reducing *de novo* sphingolipid synthesis. The regulation mechanism implies phosphorylation by kinases and phosphatases downstream of TORC proteins. Another mechanism implicated is the Ca^{2+}/calcineurin induced upregulation of Orm2. Image obtained from Carreras, 2014 [8].

Regarding the implication of human ORMDLs in *de novo* sphingolipid synthesis, a conserved role compared to yeast orthologs was detected. Firstly, expression of human ORMDL3 reverted growth arrest in yeast double-KO mutants for Orm1 and Orm2 [1,77]. Secondly, co-immunoprecipitation between ORMDL3 and SPTLC1 in the mammalian system showed a direct interaction, resulting in elevated sphingolipid levels after complete ORMDL-KO [77,154]. And lastly, ORMDL proteins are able to sense intracellular sphingolipid content, thereby confirming that the inhibitory function on SPT is mediated by a negative feedback loop also in human cell lines [154]. The three ORMDL proteins are thought to have a redundant function regarding the *de novo* sphingolipid synthesis,

since all three ORMDL isoforms must be depleted to completely block synthesis, whereas pairwise or single KO is not sufficient [154]. However, until the first scientific contribution of this thesis, the impact of overexpressing individual ORMDL proteins on SPT function has not been fully elucidated. This is important because the association to the pathophysiology implies expressional changes of the individual family member ORMDL3. Recent studies have also showed that overexpression of ORMDL3 alone did not inhibit sphingolipid biosynthesis in HeLa cells, airway epithelial cells and macrophages [155,156].

In the context of regulated expression, an upregulation of ORMDL proteins in response to high SPT enzymatic activity and the opposite effect after blocking it with myriocin was reported. This upregulation was also abolished after fumonisin treatment, a specific inhibitor of ceramide synthases. This reaction occurs downstream of SPT in the *de novo* sphingolipid synthesis, indicating that ORMDL proteins are sensitive not to *de novo* sphingoid bases, but rather ceramide or other downstream metabolites [157]. Moreover, not the ORMDL expression levels itself, but rather the stoichiometry between SPT and ORMDLs seems to determine enzyme activity [158]. Interestingly, ORMDL-SPT complex formation is not altered depending on sphingolipid levels. It was therefore suggested that ORMDLs change their conformation towards an inhibitory state in order to block sphingolipid synthesis [158]. Taken together, these studies provide further insight into the regulation of the *de novo* sphingolipid synthesis by ORMDL proteins, but this complex mechanism of interaction and regulation still needs further investigation.

2.5 Sphingolipids and disease

2.5.1 Genetic disorders

Hereditary Sensory and Autonomic Neuropathy Type I (HSAN-I) is an autosomal dominant progressive degeneration of neurons, which is mapped to mutations in the *SPTLC1* gene on chromosome 9 [159,160]. Initially, four point mutations were identified causative for the disease (C133W, C133Y, V144D, and G387A). These mutations showed a negative effect on SPT activity and sphingolipid production by interfering with the interaction site of SPTLC1 and SPTLC2, whereas SPT expression levels in lymphoblasts of HSAN-I patients remained unchanged [159–162]. Additional disease-causing mutations were also discovered in the SPTLC2 gene (V359M, G382V, and I504F) resulting in a decreased SPT activity [163,164]. SPT mutants show altered specificity for their amino acid substrate. In fact, they are able to use L-alanine or L-glycine as alternative substrates resulting in elevated levels of atypical deoxysphingoid bases, a hallmark in HSAN-I patients [165,166]. Oral supplementation with the natural substrate L-serine prevented accumulation of deoxysphingoid bases and improved HSAN1 symptoms[167].

2.5.2 Asthma

There are evidences showing that imbalance of sphingolipid homeostasis contribute to asthma pathogenesis. However, most studies of sphingolipids in asthma were focused on S1P, as elevated levels were found in bronchioalveolar lavage (BAL) fluid of asthma patients after allergen challenge [168]. Additionally, S1P has been implicated in airway hyperresponsiveness, lung inflammation, and mast cell activation [168–173]. Administration of S1P led to increased airway resistance, mast cell and

eosinophil recruitment to the lung [173], as well as mast cell migration and degranulation [174] and secretion of pro-inflammatory cytokines [175] in mice. Furthermore, C16-ceramide was increased in exhaled breath collection in asthma patients compared to healthy controls [156].

Regarding *de novo* synthesis, a decrease in this pathway has emerged as a contributing factor to bronchial reactivity, thereby providing a direct link between asthma pathology and *de novo* sphingolipid synthesis [176]. This results would provide a functional explanation to the genetic studies from Moffatt and colleagues showing that the polymorphism associated with asthma leads to elevated expression levels of ORMDL3 [10], a negative regulation of SPT. In addition, the inhibition of *de novo* sphingolipid synthesis by either myriocin treatment or haploinsufficiency of SPT showed increased airway hyperreactivity [176]. Furthermore, a transgenic mouse model was described with a stable overexpression of human ORMDL3, which also showed increased airway remodeling and responsiveness [6]. However, until the second scientific contribution of this thesis, it was not clear if this transgenic mouse model shows an impairment of *de novo* sphingolipids synthesis leading to the asthmatic phenotype.

3. Macrophages

3.1 Origin and expression

Macrophages are mononuclear, highly phagocytic cells of the innate immune system providing the first line of defense against infection. For many years detection and elimination of pathogens was thought to be the principle task of macrophages. Nowadays, there is growing evidence that they are also involved in developmental processes, maintain homeostasis (e.g. blood pressure physiology, lipid metabolism) and influence tissue repair and regeneration.

Macrophages are widely expressed in all mammalian tissues and show an immense heterogeneity due to their transcriptional profile [177]. Tissue-resident macrophages are named after their tissue location, such as osteoclasts (bone), alveolar macrophages (lung), microglial cells (CNS), histiocytes (connective tissue), Kupffer cells (liver), and Langhans cells (LC) (skin).

These macrophages originate from three different sources:

- yolk sac (giving rise to some tissue-resident yolk sac-derived macrophages)
- fetal liver (giving rise to fetal liver-derived macrophages)
- bone marrow (giving rise to tissue-resident bone marrow-derived macrophages

The origin of adult macrophages shows strong variation depending on the particular tissue and is therefore subject to ongoing discussions [178,179]. Particularly, to which extend embryonic yolk sac-derived macrophages are later replaced by the other two subsets or whether they are able to

proliferate in the relevant tissue remains unclear. However, it is well described that BMDM originate from hematopoietic stem cells located in the bone marrow and are released into the circulation as blood monocytes; the spleen is considered a storage reservoir of immature monocytes [180]. These blood monocytes are mainly patrolling the surface of the vascular endothelium in order to detect damage or pathogens [181,182]. Once they migrate from the blood circulation through the endothelium, they differentiate into macrophages. Thereby replenishing the pool of tissue-resident macrophages in steady state conditions, mainly in organs of constant necessity of macrophages (such as the uterus) or continuous exposure to microbiota (gut and skin) [183].

3.2 Role in inflammation

During early inflammation fibroblasts, epithelial cells and endothelial cells produce high levels of mainly two cytokines: CCL2 and CX3CL1, which induce inflammation-dependent recruitment of monocytes to the inflamed tissue [181,184]. Subsequently, highly abundant recruited monocytes infiltrate the inflamed tissue and get activated by interaction with pathogens or damaged cells [185]. After activation, a change in their transcriptional profile towards an inflammatory (or M1-like) phenotype occurs, which is characterized by the production of inflammatory chemokines to attract other effector cells (such as granulocytes, natural killer (NK) cells, T-lymphocytes) and the release of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) [181].

At a later stage of inflammation and in order to resolve it, additional monocytes are recruited and together with existing macrophages differentiate into cells that resemble an anti-inflammatory (M2-like) phenotype, which promote tissue repair and healing [186–188].

3.3 Functional properties

3.3.1 Pathogen recognition

The basic principle for immune recognition is the distinction between self tissue and microbial pathogens. For this reason, macrophages express a variety of pattern recognition receptors (PRRs) on their plasma membrane that recognize pathogen-specific molecular structures fundamentally different from the host, termed pathogen-associated molecular patterns (PAMPs) [189,190]. To date a large number of PRRs have been identified and divided by participation in cellular and humoral response mechanisms, which are interconnected for proper host defense [191,192]. The cellular PRR are further subdivided into intracellular (e.g. NOD-like receptors (NLR)) or cell surface molecules, of which the latter constitutes phagocytic receptors (e.g. scavenger, lectins) and sensors (e.g. Toll-like receptors (TLR)).

Phagocytic receptors bind their ligands directly and stimulate particle internalization via a rearrangement of the actin cytoskeleton. The subsequent extension of the plasma membrane leads to the formation of a phagolysosome and ultimately to the elimination of the particle. Those phagocytic receptors display molecular properties and functional kinetics of classical receptors. In contrast, sensors like TLRs do not bind their ligands directly nor induce internalization, but recognize PAMPs and trigger a pro-inflammatory signaling cascade to evoke antibiotic effector responses.

3.3.2 Macrophage polarization

The activation of macrophages after pathogen exposure has been first observed by an enhanced antibacterial activity [193]. Treatment with

supernatant from activated T-cells led to a similar activation state, which was attributed to interferon-y (INFy) [194,195]. This was the first description of the so-called classically activated macrophages or M1 macrophages, which display a pathogen killing or inhibitory capacity.

Later on, another subset of macrophages was described due to the exposure of interleukin (IL)-4 and IL-13 expressed by T helper 2 (Th2) cells. These macrophages not only differ in receptor expression on the plasma membrane and cytokine secretion [196], but also display the opposite function of M1 macrophages, namely promoting tissue healing and growth [197,198]. They were therefore named alternatively activated macrophages or M2 macrophages. The classification of M1/M2 macrophages was derived from the Th1/Th2 responses in T-lymphocytes, as macrophages from mouse strains with Th1/Th2 background showed striking differences in their arginine metabolism. M1 macrophages are primarily characterized by the production of nitric oxide (NO) and citrulline via the inducible nitric oxide synthase (iNOS) pathway, resulting in an effective pathogen killing mechanism. Whereas M2 macrophages mainly metabolize arginine via the arginase pathway to produce ornithine, a precursor for polyamines and collagen, thereby promoting cell replication and healing [199] (Fig. 12).

This simplified paradigm of M1/M2 macrophage polarization has been regrouped by Mantovani and colleagues, who classified the diverse continuum of phenotypes based on the expression of selected macrophage markers after stimulation [196]:

M1 –stimulation by INFy combined with LPS or TNFa
M2a – stimulation by IL-4/IL-13
M2b – stimulation by immune complex and TLR ligand
M2c – stimulation by IL-10, or glucocorticoid (GC)

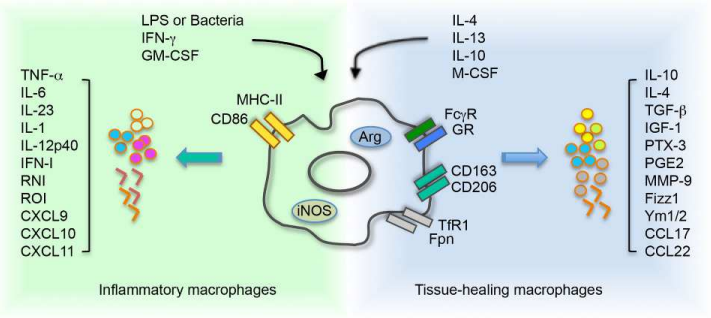

Figure 12. Schematic of macrophage polarization. Macrophages polarize towards the M1 or M2 phenotype depending on the stimuli, displaying a pro-inflammatory or anti-inflammatory profile. Image modified from Bosurgi *et al.* 2011 [200].

The complexity of macrophage subset characterization is subject to ongoing research, but has already shed light on signaling pathways of several stimuli. Nevertheless, the current classification remains challenging, since it is based on *in vitro* studies that cannot be easily translated into an *in vivo* system. Further experiments are needed to elucidate the physiological source and context of certain stimuli, the impact of multiple stimuli occurring at the same time in tissues, and the interplay between partially overlapping macrophage subsets.

3.3.2.1 M1 stimuli

The M1 macrophage activation is stimulated by exposure to intracellular pathogens, lipoproteins, bacterial cell wall components and cytokines such as IFNy and TNFa. All M1 stimuli induce similar inflammatory responses and cytokine expression profiles, but differ considerably in their source, receptor recognition and signaling pathways. Herein, the main and most relevant M1 stimuli for this thesis are discussed.

Interferon-γ (INF-γ)

The main cytokine associated to M1 activation is INF- γ, which is primarily produced by Th1 cells, but also NK cells and macrophages themselves. Its receptor is composed of IFNGR-1 (responsible for ligand binding) and INFGR-2 (responsible for signal transducing) chains and signals via the so-called JAK-STAT pathway. Upon interaction of INF-γ with the ligand binding domain of INFGR-1, the assembly of an active receptor complex takes place [201]. Leading to the activation of the Janus kinases JAK1 and JAK2, which phosphorylate a tyrosine residue at the cytoplasmic domain of INFGR-1 and thereby trigger the phosphorylation of signal transducer and activator of transcription 1 (STAT1) [202]. The transcription factor STAT1 forms homodimers and translocates to the nucleus to induce transcriptional activation of INF-y inducible genes [203]. Stimulation of peripheral blood mononuclear cells (PBMCs) with IFN-γ resulted in the induction of 111 genes, including several chemokines (CCL8, CXCL9, CXCL10, CXCL11), interleukins (IL-1A, IL-7 and IL-15) and cell surface receptors (CCR5, CD38, CSF2RB, FCGR1A, ICAM, IL15RA, MSR1, NKTR, P2RY13, SLAMF1) among others (for complete list: Waddell et al. 2010).

The combination of IFN-γ and LPS is applied in order to fully induce M1 polarization in the M1/M2 paradigm, since gene expression profiles from either LPS or IFN-γ alone differ [205,206].

Enhancement of innate immune activation (Priming)

IFN-γ enhances the response of macrophages to other inflammatory stimuli, such as TLR ligands through a process called “priming”. Interestingly, IFN-γ priming substantially increases TLR- activated signal transduction, particularly by upregulating TLR expression, promoting NfkB activation and inducing certain TLR responsive genes (reviewed in

[207]). Moreover, IFN-γ priming seems to include a distinct pathway of inactivating the TLR-induced feedback inhibition loop [208].

Lipopolysaccharide

The best-studied M1 activation signal is LPS, a membrane constituent of the outer leaflet of gram-negative bacteria, which is mainly recognized by TLR4. Structurally LPS is a glycolipid composed of a hydrophobic lipid A region, responsible for its inflammatory activity, which is attached to a long carbohydrate chain. Due to its amphipathic nature LPS forms large aggregates in aqueous environments. These aggregates show high affinity for LPS binding proteins (LBP), which can transport them to membrane-bound or soluble cluster of differentiation 14 (CD14) [209,210]. CD14 has been shown to facilitate LPS-induced signaling by binding LPS/LBP aggregates and subsequently loading them onto the TLR4-myeloid differentiation factor 2 (MD-2) complex [211,212]. LPS binding to the large hydrophobic pocket of MD-2 causes dimerization of the TLR4-MD-2 complex, which is needed for proper signaling [213]. Both TLR4 and MD-2 are crucial for macrophage activation since knockout mice of either molecule do not respond to LPS [214,215].

After TLR4 activation Toll/IL-1R homology (TIR)-containing adaptor proteins are recruited, namely myeloid differentiation factor 88 (MyD88) and MyD88 adaptor-like (Mal), which interact with the cytoplasmic TIR-domain of TLR4. In the MyD88-dependent pathway the following transcription factors undergo translocation to the nucleus: nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells (NfκB), activator protein 1 (AP-1) and CCAAT/enhancer binding protein (C/EBP), leading to the transcription of many pro-inflammatory signaling molecules, under which TNF-α is the most prominent one. On the other hand, the MyD88-independent pathway recruits the adaptor proteins TIR-domain-containing

adaptor inducing interferon-b (INF-β) (TRIF) and the TRIF-related adaptor molecule (TRAM), which induce gene upregulation of type I interferons, mainly INF-β under the regulation of the interferon regulatory transcription factor 3 (IRF-3) (Fig. 13).

LPS activation induces the transcription of 347 genes in murine bone marrow macrophages, including cytokines (INF-β, IL-12, TNF-α, IL-6, IL-1β), chemokines (CCL2, CXCL10, CXCL11), antigen presenting molecules and others [216]. The LPS-induced target genes are expressed in a temporal cascade: many pro-inflammatory cytokines are transiently induced or reduced with a peak shortly after 2-4h and get progressively repressed thereafter; whereas other genes are induced at later time points of up to 24h after stimulation, especially targeting inducible transcription factors or acting in an autocrine way [217].

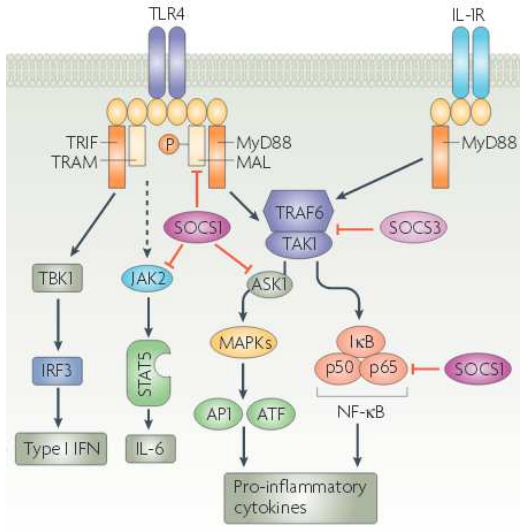

Figure 13. TLR4 signalling after macrophage activation. Activation of TLR4 by LPS induces signaling trough the adaptor proteins Myd88 and TRIF in order to trigger pro-inflammatory cytokine production. Image modified from Yoshimura *et al*. 2007 [218].

Granulocyte macrophage colony stimulating factor (GM-CSF)

GM-CSF is classified as an M1 stimulus and used to generate murine dendritic cells (DCs) and macrophages derived from bone marrow (GM-BMM) [219]. Human monocytes treated with GM-CSF show "M1-like" gene expression profiles with the induction of the transcription factors NfκB and IRF-5 [220,221]. Thus, leading to the expression of several pro-inflammatory cytokines (IL-6, IL-8, TNF-α, and IL-1β) and cell surface molecules for antigen presentation and complement- as well as antibody-mediated recognition [222].

3.3.2.2 M2 stimuli

The M2 macrophage activation is induced by stimuli originating from different recognition levels, spanning from maturation signals, interaction with other immune cells, direct pathogen interaction and its resolution. M2 stimuli provoke antagonizing effects to the inflammatory responses induced by M1 macrophages, and differ considerably in their source, receptor recognition and signaling pathways.

Interferon-4 / Interferon-13 (IL-4/IL-13)

IL-4 is the first described cytokine associated to M2 activation, which is characterized by an upregulation of macrophage mannose receptor (MMR or CD206) and major histocompatibility complex class II (MHC II), as well as reduced pro-inflammatory cytokine secretion [197]. This cytokine is produced by Th2 cells, eosinophils and macrophages and specifically recognized by its IL-4Rα receptor that pairs with the common γ-chain (γc) in order to activate JAK1 and JAK3. Pairing of IL-4Rα with the IL-13Rα1 subunit instead, enables IL-4 and also IL-13 binding to their respective subunits. The IL-13Rα1 subunit activates other kinases, namely tyrosine kinase 2 (TYK2) and JAK1/2 [223,224]. Upon receptor activation JAKs

phosphorylate the cytoplasmic tail of IL-4Rα and enable STAT-6 binding [225]. STAT-6 dimerization and nuclear translocation leads to the induction of several genes, such as eotaxin-1, arginase-1 SPRR2A, ADAM8 and others [224] (Fig. 14).

Although, IL-4 and IL-13 treatment induce similar expression profiles due to a shared receptor chain, they also elicit distinct functions in asthma disease [227,228]. Another IL-4-specific signaling pathway is induced via the insulin receptor substrate–2 (IRS-2), which activates phosphoinositide 3-kinase (PI3K) and the downstream the serine/threonine kinase Akt to promote survival and cell growth [229,230]. Furthermore, IL-13 binds specifically to its soluble and membrane-bound decoy receptor IL-13α2, which is unable to induce STAT-6 activation [231], but seems to be involved in the prevention of apoptosis in glioblastoma cells [232]. Further studies might explain the exact contribution of IL-4 and IL-13 signaling pathways involved in allergic asthma.

Macrophage-colony stimulation factor (M-CSF)

M-CSF is classified as an M2 stimulus and widely used to generate murine BMDM [233]. Human monocytes treated with M-CSF show significant features of M2 activation [205], as well as induction of cell cycle genes and repression of antigen-presenting and co-stimulatory molecules (HLA, CD80, CD40) [234,235].

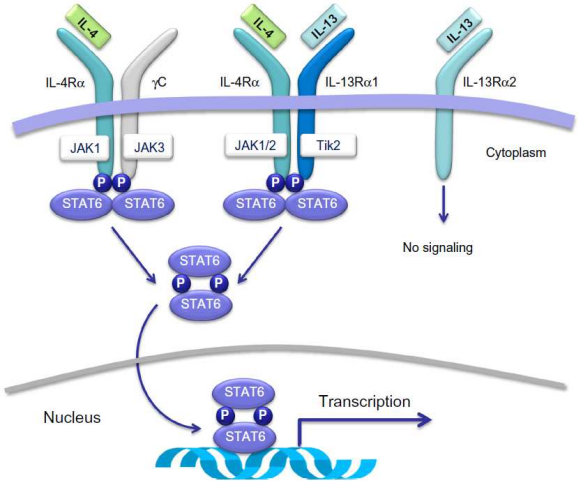

Figure 14. Signaling pathways activated by IL-4/IL-13. IL-4 and IL-13 activate a heterodimeric receptor complex consisting of the IL-4 receptor α-subunit (IL-4Rα) and the IL-13 receptor α1-subunit (IL-13Rα1). Image obtained from Vatrella *et al*. 2014 [236].

3.4 Phagocytosis

Phagocytosis is characterized by the engulfment of infectious extracellular material with a diameter above 0.5um into single-membrane phagosomal compartments, which is then degraded by proteases after fission with endosomes or lysosomes. It is thereby involved in mammalian defense against infection [237]. Phagocytosis is induced by infectious agents and apoptotic cells following stimulation of phagocytic receptors, such as scavenger receptors, TLRs and opsonin receptors. The latter have been described to increase phagocytic uptake of previously coated bacteria with immunoglobulin antibodies or those that have been marked by the complement system [238]. The internalization process is dependent on actin cytoskeleton remodeling, specifically the extension of the plasma membrane around the particle, followed by the production of superoxide and release of inflammatory cytokines from the phagocyte [239].

Interestingly, blocking the *de novo* sphingolipid synthesis with myriocin abrogates phagocytosis of fungal *candida albicans* in macrophages. Suggesting a crucial role for *de novo* sphingolipids in clearance of fungal infection at the binding stage and phagocytic cup formation [240]. The role of *de novo* sphingolipid synthesis in phagocytosis of other pathogens or bacterial infections has not been further investigated and is therefore subject of the presented thesis.

3.5 Autophagy

Macroautophagy (hereafter referred to as autophagy) is a catabolic process characterized by the lysosomal degradation of long-lived proteins and bulky cytosolic contents in order to maintain cellular homeostasis. The metabolites of lysosomal activity can be reused to synthesize new macromolecules or to control energy homeostasis for other metabolic processes. Autophagy can therefore be described as an intracellular recycling mechanism displaying cytoprotective function [241,242].

The hallmark of autophagy is the formation of so-called autophagosomes, which are double-membrane vesicles engulfing molecules for lysosomal degradation (Fig. 15).

The autophagic process consists of four main steps:

(1) autophagy initiation
(2) elongation of autophagosomes
(3) autophagosome closure
(4) fusion with lysosomes

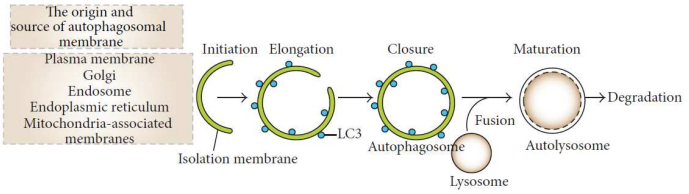

Figure 15. General scheme of the autophagic process. Initiation of the isolation membrane is followed by elongation and closure resulting in an autophagosome that surrounds the cargo. The fusion of lysosomes with autophagosomes causes the formation of autolysosomes; therein autophagic substrates are degraded. Image modified from Vural *et al*. 2014 [243].

Autophagy is generally induced under nutrient starvation via the inhibition of mammalian target of rapamycin (mTOR). The so-called autophagy-related gene (ATG) proteins, initially characterized in yeast, widely assist in autophagosomal biogenesis [244]. Starvation results in the translocation of the mTOR substrate complex UNC-51-like kinase1/2 (ULK1/2) from the cytosol to the ER [245,246]. ULK activates Beclin-1 via phosphorylation, thereby recruiting class III phosphoinositide-3-kinase (VPS34) and triggering the complex formation of VPS34-Beclin-1-Atg14 [247]. Subsequently, autophagosomes emerge in the cytoplasm starting with the formation of isolation membranes to which the active ULK and Beclin-1 complexes translocate. Isolation membranes originate from the ER, Golgi apparatus or ER-mitochondrial contact sites, supported by plasma membrane derived endocytic organelles [248,249] (Fig. 16).

The autophagic process is further mediated by two ubiquitin-like protein conjugation systems. The first system contains a covalent Atg5-Atg12 conjugate that binds Atg16L1 to form a putative E3 enzyme [250]. This complex directs the formation site of the second conjugate by binding to Atg3, which mediates the ligation of Atg8 (the most studied herein is the microtubule-associated protein light chain 3 (LC3)) covalently to the lipid phosphoethanloamine (PE) on the surface of autophagosomes [251].

Therefore, autophagy induction can be monitored by the translocation of LC3-I from the cytosol with a diffuse cytosolic distribution to newly formed autophagosomes, which appear as cytoplasmic puncta LC3-II [252]. Membrane-bound LC3-II contributes to further autophagosome elongation and closure. Its interaction with several adaptor proteins, such as p62 [253] and NBR1 [254] seems to be a possible mechanism for recognition of autophagic targets. The last step is the fusion of the autophagosome with lysosomes mediated by SNARE proteins and others. Finally, the autolysosomal content is degraded and the remaining metabolites are released by permeases [255].

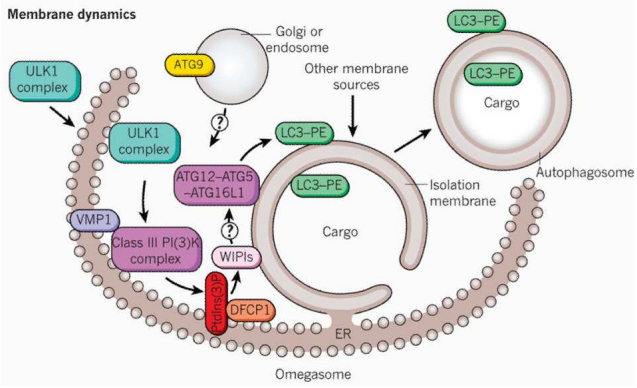

Figure 16. Schematic overview of autophagy. Overview of the autophagy pathway displays the current understanding of the molecular signaling events involved in membrane initiation, elongation and completion of the autophagosome. After induction of autophagy, the ULK1 complex activates Beclin-1 and recruits the VPS34 complex. ATG12–ATG5–ATG16L1 complex are present on the outer membrane, and LC3–PE is present on both the outer and inner membrane of the isolation membrane. Image modified from Levine *et al.* 2011 [256].

Although, this “self-eating” process has been identified more than 50 years ago [257], recent developments revealed a crucial role for autophagy in immunity and inflammation that might play a protective role in infectious, autoimmune or inflammatory diseases. Stimuli for autophagy induction include nutrient deprivation, damaged or excessive organelles, misfolded protein aggregates, ER stress, oxidative stress, certain toxins, pathogens, radiation and hypoxia [241].

3.5.1 Autophagy in macrophages

Recent studies demonstrated that autophagy is induced downstream of TLR stimulation. In this regard, the TLR4 signaling pathway upon LPS treatment is best characterized; providing a mechanistic link between pathogen recognition via PRRs and subsequent elimination through autophagy [258–261]. LPS was shown to induce autophagy via the Myd88-independent pathway adaptors TRIF and RIP1, which lead to the induction of p38MAPK thereby favoring cell survival [260]. Upon LPS treatment Beclin-1 is dissociated from its anti-autophagic binding partner Bcl-2 and interacts with VPS34. This complex participates in autophagosome formation via the recruitment of other autophagy proteins to the pre-autophagosomal membrane [262,263].

Phagocytosis and autophagy display highly conserved and interconnected cellular functions. In order to clarify the distinct contribution of each process in pathogen elimination via TLR4 signalling, Xu and colleagues proposed a cooperative model of sequential events triggered by TLR4 ligands: 1) pathogen internalization by phagocytosis and 2) fusion of phagosomes with autophagosomes and lysosomal degradation by autophagy. A previous study revealed that phagocytosis signals through the TLR4 Myd88-dependent pathway [264], suggesting a rapidly induced response after pathogen recognition. In contrast, autophagy is induced by

the TLR4-TRIF Myd88-independent pathway, which would account for a slower response and degradation [260]. An interaction between phagosomes and autophagosomes formed by the respective processes was also claimed [261,265]. More recently, induction of autophagy was shown to reduce particle internalization via phagocytosis [266], whereas its blockage led to an increased phagocytosis due to the upregulation of phagocytic scavenger receptors [267], demonstrating a clear cross-talk between those pathways (Fig. 17).

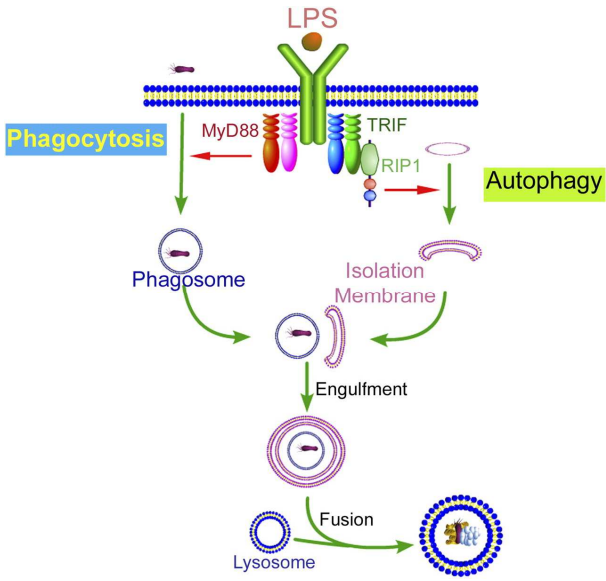

Figure 17. Schematic model of autophagy and phagocytosis interplay. Pathogens are rapidly phagocytosed via Myd-88 dependent TLR4 pathway, followed by a slower TRIF-dependent TLR4 activation of autophagy Autophagosomes encapsulate infected phagosomes before the fusion with lysosomes, leading to the effective removal of the sequestered pathogen. Image obtained from Xu *et al*. 2012 [268].

3.5.2 Autophagy and sphingolipids

Moreover, several studies have established that exogenously applied ceramide mediates autophagy [269–272]. In particular, treatment of cancer cells with C2- or C6-ceramide resulted in LC3-II puncta formation at the autophagosomal membrane via inhibition of the mTOR pathway. Increased expression of Beclin-1 and its dissociation from of the Beclin1/Bcl-2 complex was also detected [270]. Tamoxifen, a potential anti-cancer agent, was shown to increase autophagic cell death by elevating endogenous sphingolipid levels, as evaluated by the same parameters. Importantly, the tamoxifen-induced autophagic phenotype was reverted after myriocin treatment, suggesting that the sphingolipids necessary for autophagy induction are derived from the *de novo* sphingolipid pathway [270,272]. Regarding inflammation, the increase of *de novo* sphingolipids triggered by LPS stimulation of macrophages has also been associated to autophagy induction. It was postulated that dihydroceramide (DHcer) species from the *de novo* sphingolipid pathway are initiating the autophagosomal vacuole formation important for further membrane elongation [273]. Nevertheless, further studies need to evaluate the ability and mechanisms of sphingolipids to induce and regulate autophagy in distinct cellular situations, such as inflammation and cancer. Herein, ORMDL proteins might play an important role as endogenous regulators of *de novo* sphingolipid synthesis and their contribution to autophagy.

II. OBJECTIVES

General Objective

The general objective of this thesis is to study the consequences of the increased *ORMDL3* gene expression observed in inflammatory diseases by GWAS on macrophage physiology as a model of innate immunity.

Specific objectives

- The evaluation of the ORMDL-SPT complex composition and regulation in mammalian cells.
- The study of the functional implication of ORMDL proteins in macrophage activation, focusing on *de novo* sphingolipid synthesis.
- The evaluation of increased ORMDL3 expression levels on macrophage physiology using a transgenic mouse model.
- The study of the regulation generated by the disease-associated genetic determinants of *ORMDL3* expression on human monocytes.

III. RESULTS

CHAPTER 1

COORDINATED REGULATION OF THE OROSOMUCOID-LIKE GENE FAMILY EXPRESSION CONTROLS *DE NOVO* CERAMIDE SYNTHESIS IN MAMMALIAN CELLS

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CHAPTER 2

IMPAIRED *DE NOVO* SPHINGOLIPID SYNTHESIS AFFECTS AUTOPHAGY IN *ORMLD3* TRANSGENIC MICE

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Manuscript in preparation

Abstract

Increased orosomucoid-like 3 (*ORMDL3*) expression levels due to Single Nucleotide Polymorphisms (SNPs) have been associated to several inflammatory diseases, including asthma. ORMDL proteins are claimed to inhibit Serine Palmitoyltransferase (SPT), the first rate-limiting enzyme in *de novo* sphingolipid synthesis. Interestingly, sphingolipid imbalance impairs innate immune responses, particularly evident during bacterial infection. The present study addressed the involvement of disease-associated ORMDL3 in macrophage physiology using isolated Bone Marrow-Derived Macrophages (BMDM) from a ORMDL3 Knock-in transgenic mice (hORMDL3^{Rosa26}). Our results showed that ceramide content and profile was notably altered in the transgenic mouse model. Remarkably, activation of BMDM in the transgenic mouse resulted in a reduction of SPT activity as measured by *de novo* sphingolipid production of sphinganine. However, gene expression analysis of several markers showed that alteration in ORMDL3 expression levels did not affect activation neither the ability of macrophages to polarize towards the M1/M2 phenotype. We then studied phagocytosis and autophagy, both processes crucial in innate immunity and dependent on lipid membrane composition. Whereas phagocytosis of *E. coli* in transgenic macrophages was not affected by ORMDL3 overexpression, we found a reduction in autophagy, a process highly dependent of *de novo* sphingolipid synthesis. Finally, studies using isolated human monocytes ruled out the idea that the genetic *cis* elements around *ORMDL3* might cause an imbalance ORMDL regulation during activation impairing a proper SPT activity release. In conclusion, this work reinforces the importance of the coordinated ORMDL expression level regulation in order to modulate SPT activity during physiological macrophage activation, a key step for autophagy induction. However, we could not link this effect to a SNP-dependent

regulation of *ORMDL3* gene expression. Nevertheless, this work underlines the importance of ORMDL3 in sphingolipid homeostasis providing new insights about the functional link between ORMDL3 expression and asthma disease.

INTRODUCTION

The association of the orosomucoid-like 3 (*ORMDL3*) gene localized to chromosome 17q21 with childhood asthma in a genome-wide association study [1] aroused interest in this protein family comprising three members of transmembrane proteins located in the endoplasmic reticulum [2]. ORMDL1 (chromosome 2) and ORMDL2 (chromosome 12) are each located on different chromosomes and have not been linked to asthma, but share a structural homology of 80% with ORMDL3 [2]. The first asthma-associated SNP described (rs7216389) is located in a non-coding region of the adjacent gasdermin B gene (GSDMB), causing the risk allele increased *ORMDL3* expression [1]. Later on, other SNPs in the 17q21 asthma susceptibility locus in close proximity to the *ORMDL3* gene have been linked to pro-inflammatory diseases such as inflammatory bowel disease, diabetes type 1 and rheumatoid arthritis [3–8], suggesting the implication of ORMDL3 in the immune system function.

Since then, the connection between increased ORMDL3 expression levels and pathophysiology has been subject to extensive studies in order to identify possible underlying mechanisms. Our laboratory previously demonstrated the involvement of ORMDL3 in calcium homeostasis by 1) impairing sarco/endoplasmic reticulum Ca^{2+}-ATPase pump activity [9] and 2) affecting store-operated calcium entry due to a decreased calcium buffering capacity of mitochondria and calcium-dependent inactivation of the calcium release-activated Ca^{2+} channels [10], thereby negatively

affecting T-cell activation. Another field of study is the effect of ORMDL3 expression on ER stress and the three branches of the Unfolded Protein Response (UPR), which led to contradictory results [9,11]. However, the ATF6 pathway was explicitly induced in a transgenic ORMDL3 mouse model in basal conditions, as well as several characteristics of asthma such as spontaneous airway remodeling and hyperresponsiveness not related to immune dysfunction [12].

Finally, ORMDL proteins have been described as negative regulators of the rate-limiting enzyme serine palmitoyltransferase (SPT) of *de novo* sphingolipid synthesis [13,14], an important signaling pathway in inflammation [15]. This regulatory mechanism operates via the formation of a complex between ORMDL proteins and SPT by direct interaction [14,16]. Therein, the three ORMDL isoforms display a redundant function as sensors of cellular sphingolipid levels [17], detecting downstream metabolites of the *de novo* pathway [18]. Interestingly, the ORMDL/SPT complex formation itself is not altered upon changes in cellular sphingolipid levels, instead ORMDLs were proposed to undergo a conformational change to regulate SPT activity [16,19]. Considering pathophysiology, the implicit impact of the disease-associated ORMDL3 in sphingolipid synthesis was initially thought to be dispensable since simultaneous ORMDL1 and ORMDL2 knock-down had no effect on basal ceramide levels in HeLa cells [17], neither did ORMDL3 overexpression in HEK293 cells [16]. On the other hand alterations in ceramide species have been recently attributed to ORMDL3 expression levels in RAW264.7 macrophages [20].

In the context of ORMDL3 association to proinflammatory diseases we decided to explore its role in innate immunity, specifically in macrophages, where we previously described that the physiological

process of macrophage activation underlies a coordinated regulation of all three ORMDL isoforms in order to allow the induction of *de novo* sphingolipid synthesis [16]. This *de novo* production has been linked to important processes in macrophage physiology like autophagy and phagocytosis. Thus, in the present work we followed two strategies to explore whether the risk allele of the SNP associated to asthma might have phenotypical consequences in macrophage physiology. On one hand we studied the impact of higher ORMDL3 expression levels, a hallmark of risk allele carriers, on macrophage physiology *in vivo*. To elucidate this hypothesis, we used BMDM from ORMDL3 transgenic mice (Tg) that constitutively overexpress human ORMDL3 (hORMDL3) and studied physiological processes like activation, polarization, phagocytosis and autophagy. On the other hand we explored the consequences of ORMDL3 overexpression on ceramide regulation under macrophage activation in the same transgenic model. In addition we analyzed whether at the transcriptional level, the genetic factors surrounding ORMDL3 locus might alter the coordinated ORMDL regulation during activation as an attempt to understand the pathophysiology associated to this region.

Altogether, this work provides new evidences of the role of ORMDL proteins in ceramide synthesis, reinforcing the idea of a transcriptional regulation of *ORMDL* genes. In addition, our results demonstrate the consequences of an anomalous expression of ORMDL3 on ceramide homeostasis in macrophages, affecting important processes like autophagy.

MATERIALS AND METHODS

hORMDL3^{Rosa26} Mouse Generation—We generated a transgenic (Tg) mouse model that ubiquitously overexpressed ORMDL3. The targeting vector consisted of the cDNA of human ORMDL3 (hORMDL3) under the control of a CAG promotor and a floxed (*lox*P flanked) transcriptional STOP-neomycin selection cassette between the CAG promoter and the ORMDL3 cDNA (Fig. 1A). This vector was inserted within the endogenous *Rosa26* locus by homologous recombination in embryonic stem (ES) cells of a C57BL/6 WT mouse line. Herein, the presence of the negative selection marker diphtheria toxin A (DTA) reduced the isolation of non-homologous recombined cell clones. ES cells with the recombined *Rosa26* locus were injected into blastocytes. Heterozygous conditional *Rosa26* knock-in mice were crossed with Cre recombinase expressing mice in order to generate hORMDL3^{Rosa26} mice. Cre recombinase activity led to the excision of the STOP cassette and recombination at the *lox*P recognition sites, resulting in the activation of ubiquitous ORMDL3 expression in mice.

Murine Bone Marrow-Derived Macrophage (BMDM) Isolation and Differentiation— Bone marrow cells were obtained by flushing the femurs from 6-8 week old C57BL/6 WT or transgenic hORMDL3^{Rosa26} mice with ice-cold PBS. Cells were differentiated in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 30% L929 supernatant containing macrophage colony stimulating factor (M-CSF), 20% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37ºC and 5% CO_2 for 7 days. L929 cell-conditioned medium was prepared by growing L929 cells in DMEM supplemented with 10% fetal calf serum for 7 days. The M-CSF

containing medium secreted by the L929 cells was harvested and passed through a 0.22-mm filter.

Healthy Control Samples—Healthy control samples were obtained from local healthy volunteers. Clinical investigation was conducted according to the Declaration of Helsinki principles. The work in this study was approved by the Bromley Research Ethics Committee and the local Ethics Committee of the AMC. Written informed consent was received from participants prior to inclusion in the study.

Human Monocyte Isolation and Genotyping—Peripheral blood mononuclear cells (PBMC) were isolated by using density gradient centrifugation (Lymphocyte separation media, PAA). Purification of cell subsets was performed by magnetic cell separation according to the manufacturer's instructions (Miltenyi Biotec) and purity was determined by flow cytometry. Human monocytes with an average purity >95% were isolated by positive selection using anti-CD14 microbeads and cultured at 37°C and 5% CO_2 in RPMI-1640 medium (Gibco), supplemented with 1% penicillin/streptomycin, 1% L-glutamine (Gibco) and 10% heat-inactivated fetal calf serum (Sigma). For genotyping of SNP rs7216389, extraction of genomic DNA (10^6 cells per donor) was performed with the Nucleo Spin Tissue Kit from Macherey-Nagel. Gene-specific human primers used to amplify the surrounding region were 5'-GTGCCTGGCATACATTCTAACTGC-3' and 5'-AGCCCTGCCTCCAAAACCTAG-3' and Biotaq DNA Polymerase (Bioline). PCR conditions were 95 °C for 4 min, 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 72 °C for 7 min with 35 cycles of amplification using 200 ng of genomic DNA. The PCR product was purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), sequenced with the Applied Biosystems BigDye terminator v3.1 sequencing chemistry and run on an ABI 3100 (Applied

Biosystems) genetic analyzer. The sequences were analyzed using DNAstar Lasergene 11 software.

In Vitro Activation and Polarization—Adherent BMDM and human monocytes (10^6 cells per condition) were activated by incubation of 100 ng/mL *Escherichia coli* (055:B5) lipopolysaccharide (LPS) for the indicated time points. For autophagy experiments BMDM were additionally incubated with 0.1 μg/mL Bafilomycin A (Sigma) for the last 2 h of LPS treatment to block autophagosomal fusion with lysosomes and protein lysates were analyzed by Western Blot.

For induction of M1 macrophages, cells were exposed to 100 ng/mL LPS and 30 ng/mL INFγ. Accordingly, M2a macrophages were obtained by treatment with 10 ng/mL IL-4 or additionally with 10 ng/mL IL-13 for the indicated time points. Total RNA or protein lysates were prepared from cells and frozen at -80 °C until analysis.

Quantitative Real Time PCR Analysis—Total RNA of BMDM was extracted using the Nucleospin RNA II kit (Macherey-Nagel) following the manufacturer's instructions. Total RNA (0.5 μg) was reverse transcribed to cDNA using the SuperScript-RT system (Invitrogen). Quantitative RT-PCR was performed on an ABI Prism 7900HT (Applied Biosystems) with SYBR Green (SYBR Green Power PCR Master Mix, Applied Biosystems).

Gene-specific mouse primers used were:

Gene of interest	Forward primer (5' – 3')	Reverse primer (5' – 3')
ORMDL1	GCATCCCCTTCTGCAGTGTT	CGGAGTCTCAAAAGGCGTTC
ORMDL2	CGTCATCCATAACTTGGCAAT	AACTGTAGTCCATAGTCCATC
ORMDL3	CTGCTGAGCATTCCCTTTGT	CACGGTGTGCAGAAAGATGT
TNF-α	GACCCTCACACTCAGATCATCTTC	CGCTGGCTCAGCCACTCC
IL-6	GCCTTCTTGGGACTGATGCT	TGCCATTGCACAACTCTTTTC
IL-1β	TGCCACCTTTTGACAGTGATG	AAGGTCCACGGGAAAGACAC
iNOS	TCACCTTCGAGGGCAGCCGA	TCCGTGGCAAAGCGAGCCAG
Arg1	GATTATCGGAGCGCCTTTCT	CCACACTGACTCTTCCATTCTT
CD206	CTGCAGATGGGTGGGTTATT	GGCATTGATGCTGCTGTTATG
MMP9	GTCTTTGAGTCCGGCAGACA	CCAGTACCAACCGTCCTTGA
ADAM8	AACAAGCAGCGTCTACGAGC	TCTCGGAGCCTTTCGGTAGA
CCR2	TCATCTGCAAAAACAAATCAAAGGA	TAGTCATACGGTGTGGTGGC
CCL20	CGACTGTTGCCTCTCGTACA	GAGGAGGTTCACAGCCCTTT
CXCL11	AGGAAGGTCACAGCCATAGC	CGATCTCTGCCATTTTGACG
β-Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG
GADPH	TGTCGTGGAGTCTACTGGTGTCTT	TGGCTCCACCCTTCAAGTG

Gene-specific human primers used were:

Gene of interest	Fw primer	Rv primer
ORMDL1	commercially obtained from QuantiTect Primer Assay (Qiagen)	
ORMDL2		
ORMDL3		
TNF-α	AGCCTCTTCTCCTTCCTGATCGTG	GGCTGATTAGAGAGAGGTCCCTGG
GsdmB	ACATGGAGGACCCAGACAAG	CACAGAGAATTCGTGCCTCA
β-Actin	ACGAGGCCCAGAGCAAGAG	GGTGTGGTGCCAGATTTTCTC

PCR conditions in all cases were 95 °C for 5 min, 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min with 40 cycles of amplification.

Ceramide Quantification—BMDM were treated for 4 h with 500 μM palmitate (Sigma) complexed in 0.5% fatty acid-free BSA (Sigma), and BSA alone was used as a control. Exposure of BMDM to 10 μM Myriocin (Myr, Sigma) for 30 min before treatment was used to block SPT activity. On the other hand side, BMDM were activated by 100 ng/mL LPS for the indicated time points. Cells after either treatment were washed twice with 1x PBS and centrifuged at 1800 rpm for 5 min at 4 °C and the pellet was

frozen in liquid nitrogen for ceramide quantification. Lipid extraction and processing was performed as reported previously [21,22]. Lipid analysis was carried out by ultraperformance liquid chromatography coupled to time-of-flight (TOF) mass spectrometry in positive electrospray ionization mode. Instrument conditions were set as in previous studies [21,22].

Western Blot—Total protein was detected by Western blot after separation on a 4–12% gradient polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membrane. Immunodetection was carried out using rabbit antibodies against ORMDL (1:1000), SPTLC1 (1:1000), SPTLC2 (1:1000), SERCA2b (1:1000), LC3 (1:500), p62 (1:1000), Beclin-1 (1:500) and mouse anti-β-Actin (1:3000) all from Abcam. Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (1:3000; GE Healthcare). The immunoreactive signal was detected by SuperSignal West Chemiluminescent substrate (Pierce) and visualized using the Molecular Imager Chemidoc XRS system (Bio-Rad). Protein extraction from murine tissues or BMDM were washed with 1x PBS, lysed in 60 µl of lysis buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-HCl, 1x Complete protease inhibitor) for 20 min on ice with agitation, and then centrifuged at 13,000 rpm at 4 °C for 30 min. Protein concentration in the supernatant was determined using the BCA Assay (Pierce). Equal amounts of protein (30 µg for BMDM and 100 µg for tissues) were loaded into each lane and were separated on an SDS polyacrylamide gel (4-12%).

Fluorescence Microscopy—Briefly, 2 x 10^5 BMDM were seeded on glass coverslips after differentiation. Basal levels of ORMDLs, LC3 and ATF6 were detected by immunofluorescence, whereas nuclear translocation of ATF6 was induced by 1 µg/µL Tunicamycin for 30 min in the control condition. Macrophage infection was performed using a red fluorescent-tagged *E. coli* strain (kindly provided by Dr. A. Valledor) and

phagocytosis assays were carried out for 30 min at 37°C and 5% CO_2 at a multiplicity of infection (MOI) of 1:5 and 1:20. Cells were then rinsed four times with ice-cold PBS and incubated with Alexa Fluor 488 Phalloidin (Molecular Probes) for 20 min on ice. The viability and concentration of bacteria was routinely verified by plating serial dilutions on ampicillin-containing agar plates and counting colonies to determine the CFU/mL. After treatment, cells were fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.1% Triton-X100 and incubated for 2 h at RT in a hydration chamber with rabbit antibodies against ORMDL (1:500; Abcam), LC3 (1:1000; Sigma) or ATF6 (1:200; Abcam). After primary antibodies cells were incubated with 1:2000 goat anti-rabbit Alexa Fluor 488 (Life Technologies) for 1 h at RT. Nuclei were stained with TO-PRO-3 Iodide (1:1000; Sigma) in PBS for 10 min before mounting in Mowiol. Digital images were taken at a Leica TSC SP confocal microscope and analyzed with ImageJ. The number of phagocytosed bacteria per cell was counted in comparison to non-infected cells. For colocalisation studies of nuclear translocation of ATF6 the JaCoP-plugin was used to obtain the Pearson and Manders coefficient.

RESULTS

Generation of transgenic hORMDL3^{Rosa26} mice

To study the causes of the genetic association between ORMDL3 and inflammatory diseases we have generated a mouse model described in Figure 1*A* and the methods section overexpressing the human ORMDL3 protein. The ubiquitous expression of ORMDL3 in our Tg mouse model was confirmed by Western Blot in a variety of tissues. Apart from brain, which shows nearly identical expression as WT mice; thymus, spleen and lung show a moderate increase in ORMDL3 expression, whereas testis and colon are tissues with a very high expression (Fig. 1*B*).

We characterized BMDM from our Tg mouse model to study the impact of ORMDL3 on the innate immune system. The detection of human ORMDL3 expression showed an ER pattern as expected (Fig. 1*C*) with a 3-fold higher ORMDL expression than observed in WT mice. It is likely that the difference compared to the endogenous ORMDL3 expression level is higher since there is no commercial antibody available that discriminates among the different ORMDL family members (Fig. 1, *D and E*). Moreover, we checked the expression of possible interacting proteins, but there were no major changes in the expression of the SPT complex subunits nor the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) (Fig. 1, *D and E*).

Macrophage activation in hORMDL3 ^{Rosa26} mice

Macrophage activation is a crucial step in the onset of the innate immune response. Therefore we compared LPS-induced changes in the gene expression profile of BMDM from WT versus Tg mice and several activation markers were monitored over time. The selected cytokines

TNF-α, IL1-β and IL-6, as well as the inducible enzyme nitric oxide synthase (iNOS), showed an early upregulation at 4h that correlates with the production of pro-inflammatory cytokines and reactive nitrogen oxide followed by a general downregulation at later times (24h) (Fig. 2, *A-D*). However, the induction and sequential regulation of pro-inflammatory cytokines after macrophage activation with LPS was not altered by increased ORMDL3 expression levels.

M1/M2 polarization in hORMDL3 ^{Rosa26} mice

We wanted to further explore the possibility that the inflammatory profile genetically associated to ORMDL3 expression levels might be explained by an imbalance of the macrophage polarization process. The combination of LPS and INF-γ is widely used to potentiate pro-inflammatory cytokine production and to polarize macrophages towards the so-called M1 phenotype [23]. As can be seen in Figure 3 *A* and *B* the expression levels of the M1 markers iNOS and IL-1β were increased compared to LPS treatment alone. Regardless of ORMDL3 levels, the same transcriptional pattern of an early induction and later decrease of those markers was detected, enabling the cells to equally polarize towards M1 macrophages. On the other hand side, IL-4 and IL-13 are well-described inducers of the anti-inflammatory M2 phenotype. In addition, ORMDL3 transcription has been described to be under the control of STAT6 pathway in epithelial cells [11]. However, no changes in ORMDL3 expression levels neither upon IL-4 treatment of BMDM, nor upon its combination with IL-13 were observed (data not shown). As expected, IL-4 treatment alone led to an increase of Arginase-1 (Arg-1) and mannose receptor CD206 expression (Fig. 3, *C and D*), which was further provoked by the addition of IL-13 (Fig. 3, *E and F*). Nevertheless, neither approach showed alteration of the polarization state towards the M2 phenotype depending on ORMDL3. In conclusion, BMDM from hORMDL3^{Rosa26} mice display an equal

transcriptional profile after exposure to extracellular stimuli like LPS/INF-γ and IL-4/IL-13 as WT cells without impairment of the polarization process towards M1 or M2 macrophages.

De novo sphingolipid synthesis in hORMDL3 ^{Rosa26} mice

ORMDL proteins have been described as negative regulators of SPT, which is catalyzing the first rate-limiting step of *de novo* sphingolipid synthesis [14,17]. Interestingly, a significant decrease in the basal sphingolipid content in BMDM from hORMDL3^{Rosa26} compared to WT mice was detected. A detailed analysis revealed that the entire range of analyzed ceramide species was reduced, with a particularly strong reduction in the long-chain species C24:0 and C24:1 (Table 1). In order to specifically address SPT activity in hORMDL3^{Rosa26} mice, since a complex enzymatic network controls overall cellular sphingolipids, we evaluated ceramide production after the addition of the enzymatic substrate palmitate (PA). The total ceramide levels were strongly increased after 4h-incubation of 500μM PA conjugated with BSA compared to control cells treated with BSA alone (Fig. 4, *A*). Herein, the most abundant ceramide species after PA induction was C16 ceramide. The pre-treatment with myriocin (Myr), a known inhibitor of SPT, abolished the ceramide production; further confirming that ceramide is mostly being produced via the *de novo* synthesis pathway. Importantly, BMDM of hORMDL3^{Rosa26} mice showed a mild non-significant reduction of total ceramide levels compared to cells from WT mice (Fig. 4, *B*), suggesting partial but not complete impairment of SPT activity in accordance to our previous studies transfecting ORMDL3 in HEK293 cells [16].

Ceramide production during activation in hORMDL3^{Rosa26} mice

It has been shown that macrophage activation by LPS leads to an increase in intracellular ceramides [24–27], which mainly originate from the *de novo* pathway at early time points and from other sources later on [27,28]. We have previously demonstrated that the mechanism underlying this increase is based on a time-dependent downregulation of all three ORMDL isoforms after LPS activation in RAW264.7 macrophages [16]. Our experiments in BMDM confirmed a reduced endogenous ORMDL expression at early time points (4h), whereas all three isoforms reached their initial expression at later times of activation (24h) both at the transcriptional and translational level (Fig. 4, *C and D*) under LPS activation. In this context, we hypothesized that stable ORMDL3 overexpression during macrophage activation might impair the release of SPT activity. To test this hypothesis, BMDM of WT and hORMDL3^{Rosa26} mice were treated with LPS for various time points and the sphingolipid content was monitored. As expected, total ceramide increased steadily over the time course of LPS activation in WT cells reaching its maximum after 24h (Fig. 4, *E*). On the contrary, in macrophages from hORMDL3^{Rosa26} mice we did not observe a significant increase in ceramide levels until 24h after the LPS treatment. The pattern of sphinganine is of particular interest, since it directly correlates to SPT activity as an early intermediate metabolite of the *de novo* sphingolipid pathway. Macrophages from WT mice display a peak at 4h after LPS activation and a latter decrease (Fig. 4, *F*). In comparison, macrophages from hORMDL3^{Rosa26} mice exhibit a reduction of SPT activity as measured by significant lower levels of sphinganine than in WT cells (Fig. 4, *G*).

Phagocytosis in hORMDL3^{Rosa26} mice

The alteration in membrane composition and the impaired SPT activity during bacterial activation caused by the overexpression of ORMDL3 led us to explore other relevant processes for macrophage physiology, in which cellular membranes play an important role. First, we tested possible modifications of phagocytosis in macrophages of hORMDL3^{Rosa26} mice. To monitor the phagocytic engulfment of pathogens, BMDM were treated with fluorescently tagged *E. coli* for 30 min at different multiplicity of infection ratios of 1:5 and 1:20, respectively (Fig. 5, *A* and *B*). Changes in bacterial uptake between hORMDL3^{Rosa26} and WT cells were negligible after this short incubation time, as assessed by measuring intracellular bacterial staining for both *E. coli* concentrations (Fig. 5, *C*).

Autophagy in hORMDL3^{Rosa26} mice

Another membrane associated process linked to *de novo* sphingolipid synthesis in macrophage physiology is autophagy [27], due to the need of sphingolipids for autophagosome formation in order to degrade internalized components promoting cellular energy homeostasis. LPS treatment leads to the formation of autophagosomes via the TLR4 signaling pathway [29], which can be monitored by the early recruitment of Beclin-1 that activates the class III phosphoinositide-3-kinase (VPS34) complex leading to autophagosome iniation [30]. The most prominent autophagy related protein is light chain 3 (LC3), which is cleaved from its initial cytosolic LC-3 I form (18 kDa) to lipidated and autophagosomal membrane-associated LC3-II (16 kDa) during the elongation of the autophagosomal membrane [31]. Following treatment of BMDM from hORMDL3^{Rosa26} and WT mice with LPS for 8h, expression levels of Beclin-1 and p62 were induced compared to basal conditions to the same extent (Fig. 5, *E*). The autophagic flux was measured by the conversion of

LC3-I to LC3-II and latter degradation in cells treated with LPS only. Addition of Bafilomycin A (Baf), an inhibitor of autophagosome and lysosome fusion, blocks the degradation of LC3-II, thereby allowing its accumulation. As can be seen in Figure 5*F* accumulation of the cleaved LC3-II is significantly lower in activated macrophages from Tg mice compared to WT cells after Baf addition.

Regulation of ORMDLs in human monocytes

Next, we decided to explore the relevance of the expressional regulation of ORMDLs under activation in human monocytes. For this purpose, blood monocytes of 20 healthy donors were exposed to LPS for various time points. According to our previous results in murine models, all three ORMDL isoforms showed a decrease after early activation (2 and 4h), of which *ORMDL1* and *ORMDL3* were mostly affected (Fig. 6, *A*). During the later stage of activation (24h), *ORMDL1* and *ORMDL3* reached their initial expression levels, whereas *ORMDL2* showed an almost 5-fold increase (Fig. 6, *A*). Simultaneously, proper macrophage activation was assured by monitoring the induction of TNF-α expression (data not shown). However, there are emerging evidences that the disease-associated SNP does not only alter the expression of *ORMDL3*, but also affects nearby genes of the chromosomal region 17q21 [32,33]. For this reason we monitored the expression of the adjacent gene *GSDMB*, which remained stable during monocyte activation. In reference to *GSDMB*, we observed a significant decrease of *ORMDL3* (Fig. 6, *B*), suggesting that the gene regulation of the ORMDL family is specifically linked to sphingolipid synthesis.

Finally, comparing basal *ORMDL3* expression depending on the asthma-associated SNP there are no differences between the genotypes (Fig. 6, *C*). When we separated the regulatory effect of activation on the *ORMDL3*

gene we could not find differences at early time points of activation, only after 24h the *ORMDL3* expression of T-allele carriers reached its initial value, whereas the expression of C-allele carriers remained at a significant lower level (Fig. 6, *D*).

DISCUSSION

Multiple SNPs in the *ORMDL3*-containing chromosomal region 17q21 have been associated to the risk of developing proinflammatory diseases, such as asthma, ulcerative colitis, Crohn's disease or rheumatoid arthritis by genome-wide association studies [1,4,7,3]. Genetic variation at SNP rs7216389 (T allele) increases *ORMDL3* expression, which was assumed to influence cellular physiology of the immune system thereby contributing to the associated inflammatory pathologies. In order to resemble the disease state, most previous studies utilized a heterologous overexpression system in various cell types including Jurkat T cells, eosinophiles and RAW264.7 macrophages [10,34,35]. Besides, recently Miller et al. characterized a transgenic mouse model with conditional *ORMDL3* overexpression, which showed several alterations in murine lung epithelium, displaying asthma-related parameters spontaneously [12]. In the present work, our main objective was to further characterize the impact of ORMDL3 on the immune system focusing on macrophage physiology and sphingolipid homeostasis. This is of particular relevance, since we previously correlated the regulation of the ORMDL proteins with an increase in *de novo* ceramides during physiological macrophage activation.

As a working model in this study we used BMDM from transgenic mice in which we have introduced the human *ORMDL3* gene into the

endogenous *Rosa26* locus. Therefore we were able to circumvent possible artifacts caused by transient transfection in sensitive processes like macrophage activation. We observed a general increase of protein expression by monitoring a variety of tissues including lung, spleen and BMDM and validated the correct targeting by localizing the protein to the endoplasmic reticulum. Recently, cytokine expression and ceramide content have been shown to depend on the amount of ORMDL3 expression ranging from moderate to high levels [20], which raises the question of its physiological levels. However, it is difficult to establish the exact amount of ORMDL3 protein produced by the risk allele as predicted in genetic studies, because the influence of the genetic component surrounding the gene has been shown to depend on the cell type [1,32,36,37]. Regarding our model, the protein induction of more than three fold in macrophages can be considered as higher levels, since commercial antibodies do not distinguish among the three ORMDL isoforms. Besides, excessive overexpression of proteins in the endoplasmic reticulum can promote ER stress and lead to collateral phenotypes triggering the unfolded protein response. Taking this into consideration, in the other transgenic model neither the insertion locus nor the amount of protein expression is controlled, resulting in an activation of the ATF6 UPR branch [12]. This effect could also explain the discrepancies in the expression of SERCA2b and metalloproteases between both models together with the usage of different cell types, such as BMDM and alveolar macrophages.

Macrophages are the first line of defense and display multiple pathogen-associated molecular patterns (PAMPs) and receptors on their cell membrane in order to detect pathogens and cytokines expressed by other cells of the immune system. In this scenario, two different populations of macrophages have been described regarding a proinflammatory profile

(M1) or the homeostatic-repairing function (M2). For this reason we have explored functional consequences of higher ORMDL3 expression levels on macrophage activation and M1/M2 polarization. This information is especially important since chronic diseases like asthma have been associated to alterations in temporal and spatial pattern of inflammatory gene expression and imbalanced M1/M2 subsets [38]. Our results demonstrate that BMDM from transgenic mice follow the same transcriptional profile after LPS activation as WT cells. This finding is in agreement with the work from Oyeniran et al. with moderate ORMDL3 expression levels in the RAW264.7 cell line [20]. On the other hand, the polarization towards M1 or M2 was not affected by ORMDL3 expression, as BMDM from both mice showed similar gene expression patterns of selected markers. Taken together, these findings argue against the hypothesis that high ORMDL3 expression produces temporal and spatial alterations of macrophage activation or polarization. Interestingly, the cytokines IL-4 and IL-13 used to induce the anti-inflammatory M2 phenotype have been attributed to an increase of *ORMDL3* expression levels in bronchial epithelium of WT mice after intranasal administration [11]. In our hands, *in vitro* stimulation of differentiated BMDM from WT mice with IL-4 or the combination of IL-4 and IL-13 did not change ORMDLs expression levels as assessed by quantitative RT-PCR (data not shown), suggesting that this pathway might be cell type-specific or mediated via a downstream mechanism of cytokine administration.

Since the first description of ORMDL proteins as intracellular sphingolipid sensors and negative regulators of the rate-limiting enzyme of *de novo* sphingolipid synthesis, a lot of studies aimed to elucidate the underlying mechanism. Nevertheless, the regulation has not been addressed *in vivo* yet. This question is of particular interest in order to clarify whether SNPs that exert a *cis* regulation leading to increased levels

of *ORMDL3* expression, are related to the specific function of this protein in sphingolipid synthesis. The impact of ORMDL3 on basal sphingolipid levels has been evaluated by knock-down studies of ORMDL1 and ORMDL2 in HeLa cells [17] and transient ORMDL3 overexpression in HEK293 cells [16], but was determined insignificant in either approach. Only recently the ORMDL3 expression level was attributed to significant alterations of ceramide species in RAW264.7 macrophages [20]. It has been shown that higher levels of ORMDL3 lead to an inhibition of SPT activity as measured by decreased *de novo* sphingolipids, especially with a C16-, C22-, and C24-backbone. Our results in BMDM from transgenic mice clearly demonstrate that increased ORMDL3 expression affects cellular ceramide homeostasis, confirming its importance in macrophage physiology. Thereby, the reduction in rather long-chain ceramide species might be caused by a more complex scenario, in which ORMDL3 might also regulate the activity of certain ceramide synthases downstream of SPT. The induction of SPT activity using palmitate is not significantly modified in the transgenic mouse, confirming that overexpression of ORMDL3 does only partially block SPT when stimulating the *de novo* ceramide synthesis, in accordance with previous results in HEK293 cells, where the overexpression of all three ORMDL isoforms was necessary to inhibit SPT activity [16]. This reinforces our idea that additional regulatory mechanisms apart from expression levels play a role for the inhibitory effect of ORMDLs on SPT depending on cellular ceramide levels. Thus, ORMDL3 is able to lower the homeostatic content of ceramides in macrophages, but not sufficient to effectively block SPT activity. This result further questions the validation of studies when myriocin treatment has been used to mimic the pathophysiological role of ORMDL3 [39].

The release of SPT activity under macrophage activation has been emphasized in the present work. ORMDL3 overexpression in transgenic

macrophages is under the control of an exogenous promotor resisting the coordinated downregulation that releases the enzymatic activity, a modulation that has been further confirmed herein using BMDM and human monocytes. As expected, we observed an inhibitory effect of ORMDL3 on *de novo* ceramide synthesis upon LPS stimulation. This decrease can be attributed to the blockage of SPT activity, as indicated by the reduction in sphinganine production. In conclusion, our work shows that impairment in the coordinated downregulation of ORMDLs prevents SPT activity release in response to bacterial encounter.

To further explore the possible consequences of altered membrane composition at basal conditions and after LPS induction caused by ORMDL3 overexpression we monitored the two membrane-sensitive processes: phagocytosis and autophagy. Interestingly, myriocin treatment was shown to inhibit phagocytosis of fungal *Candida albicans* in macrophages, suggesting a crucial role for *de novo* sphingolipids in clearance of fungal infection at the binding stage and phagocytic cup formation [40]. In our model, phagocytosis was not impaired by ORMDL3 expression after a rather short pathogenic *E. coli* incubation time of 30 min. On the other hand, autophagy induction after LPS exposure has been shown to be highly dependent on *de novo* ceramide synthesis [27]. In this sense, we have observed that increased ORMDL3 expression impairs autophagy induction, specifically at the level of autophagosome elongation since LC3-II but not Beclin-1 was reduced in the transgenic mouse. The autophagic impairment occurs after a moderate incubation time of 8h, which correlates exactly with the time window of *de novo* sphingolipids synthesis. Dysregulation of autophagy after bacterial infection might lead to a negative outcome of bacterial clearance and thereby sustained inflammation [41]. Indeed, one of the known causes of inflammatory bowel diseases, to which *ORMDL3* has been also

genetically associated [4,5,3], is an anomalous autophagic process in the gut [42,43].

Finally, considering the interference with the normal gene expression regulation of ORMDLs under macrophage activation in our transgenic mice, we decided to explore whether this regulation occurred in humans and more interestingly, whether it was affected by the *cis* regulatory elements of the *ORMDL3* locus. In this respect, we demonstrate that human monocytes also show a coordinated regulation of all three ORMDL isoforms under physiological stimuli. Furthermore, we can attribute this regulation to the ceramide synthesis since the expression of gasdermin B (GSDMB), a gene in close proximity to *ORMDL3* and co-regulated by the surrounding SNPs, was not affected by LPS activation. We thereby confirm the unique role of ORMDL3 in monocyte activation, while strengthening our hypothesis in which specific ORMDLs regulation is important for proper functioning of the sphingolipid synthesis. On the other hand, neither ORMDL3 basal expression levels nor repression under activation were altered depending on the risk allele at SNP rs7216389 in monocytes. The lack of influence of basal expression could be due to the sample size. However it is also likely that other transcriptional determinants might overcome the ones analyzed depending on the cell type.

Our work reinforces the current view of ORMDLs as negative modulators of SPT activity and their importance specifically in macrophage physiology. Regarding the general idea of association between increased ORMDL3 expression and a higher risk to develop inflammatory pathologies, our transgenic mouse model showed reduced ceramide content and altered *de novo* synthesis under macrophage activation. These effects resulted in impairment of autophagy, an alteration that could contribute to further understanding ORMDL3 pathophysiology. However,

our data with human monocytes showed that the coordinated ORMDL downregulation is not dependent on the genetic components around the *ORMDL3* locus challenging the contribution of macrophages in ORMDL3-associated pathophysiology. Nevertheless, this work gives a better understanding of the role of ORMDLs in macrophage physiology and provides further insight on how to design and analyze future experiments.

TABLE 1

	WT	Tg
C14	3,8±1,3	3,5±1,0
C16	123±19,2	101,3±11,2
C16:1	7,9±2,4	7,1±2,3
C18	12,5±4,4	6,7±2,2
C20	7,8±2,0	5,1±1,9
C22	70,3±9,9	48,3±9,4
C24	154,6±15,8	90,2±10,8[a]
C24:1	152,9±13,8	98,8±11,6[a]
C24:2	30,7±8,4	13,2±4,6
Total	563,5±55,2	374,1±44,6[b]

[a] $p < 0.01$
[b] $p < 0.05$

TABLE 1: Ceramide content depending on ORMDL expression levels Ceramide content was quantified by mass spectrometry in BMDM from Wild-type (WT) and transgenic (Tg) mice after 7 days of differentiation with MCS-F (n=7). Data expressed in pmol/1 x 10^6 cells.

FIGURE 1

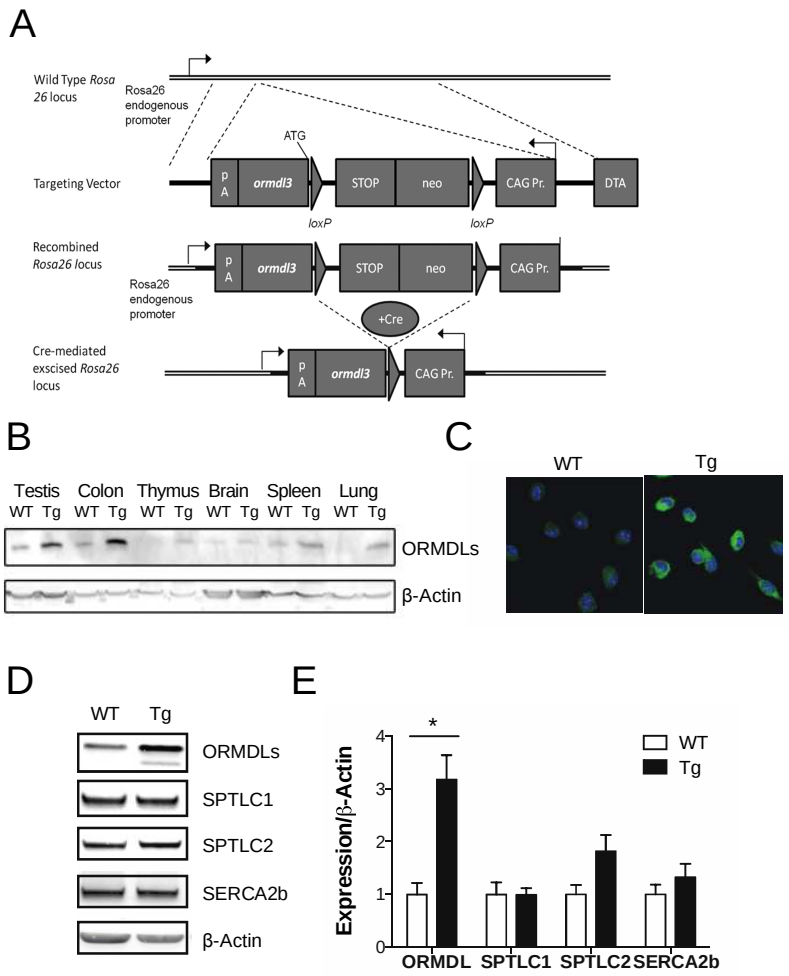


FIGURE 1. Generation and characterization of transgenic hORMDL3^{Rosa26} mice. *A*, strategy for a constitutive ORMDL3 expressing mouse model within the *Rosa26* locus. *B*, Western blot using 100µg of protein from different tissues. *C*, Immunofluorescence of ORMDL3 in BMDM from WT and hORMDL3^{Rosa26} (*Tg*) mice *C* and *D*, Representative western blot and protein expression quantification of ORMDLs, SPTLC1, SPTLC2 and SERCA2b from BMDM of WT and hORMDL3^{Rosa26} (*Tg*) mice normalized to β-Actin ($n = 4$; *, $p < 0.05$). *Error bars* represent S.E.

FIGURE 2

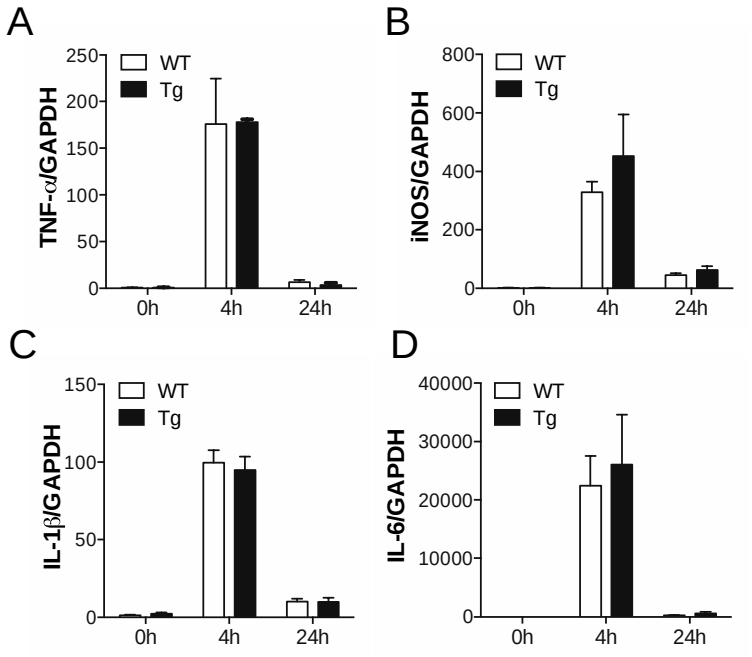


FIGURE 2. Analysis of the macrophage activation process. Gene expression analyzed by RT-PCR after activation of BMDM from WT and hORMDL3^{Rosa26} mice with 100 ng/mL LPS at indicated time points. *A*, TNF-α, *B*, iNOS, *C*, IL-1β and *D*, IL-6. Data are normalized to GAPDH (n = 6-8; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* represent S.E.

FIGURE 3

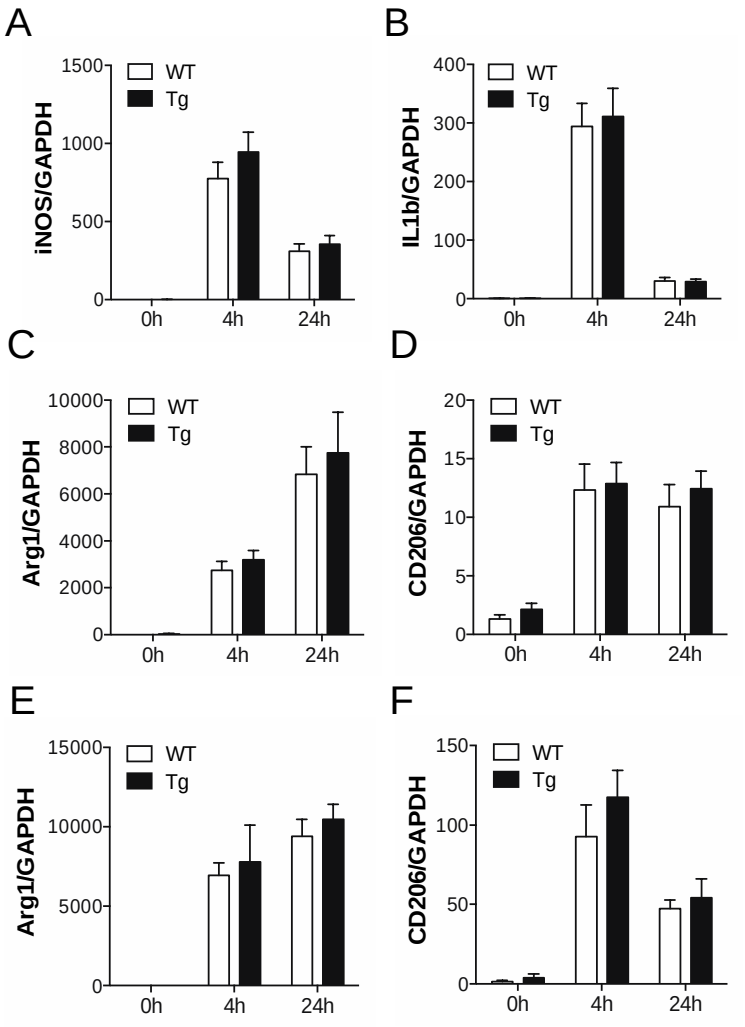
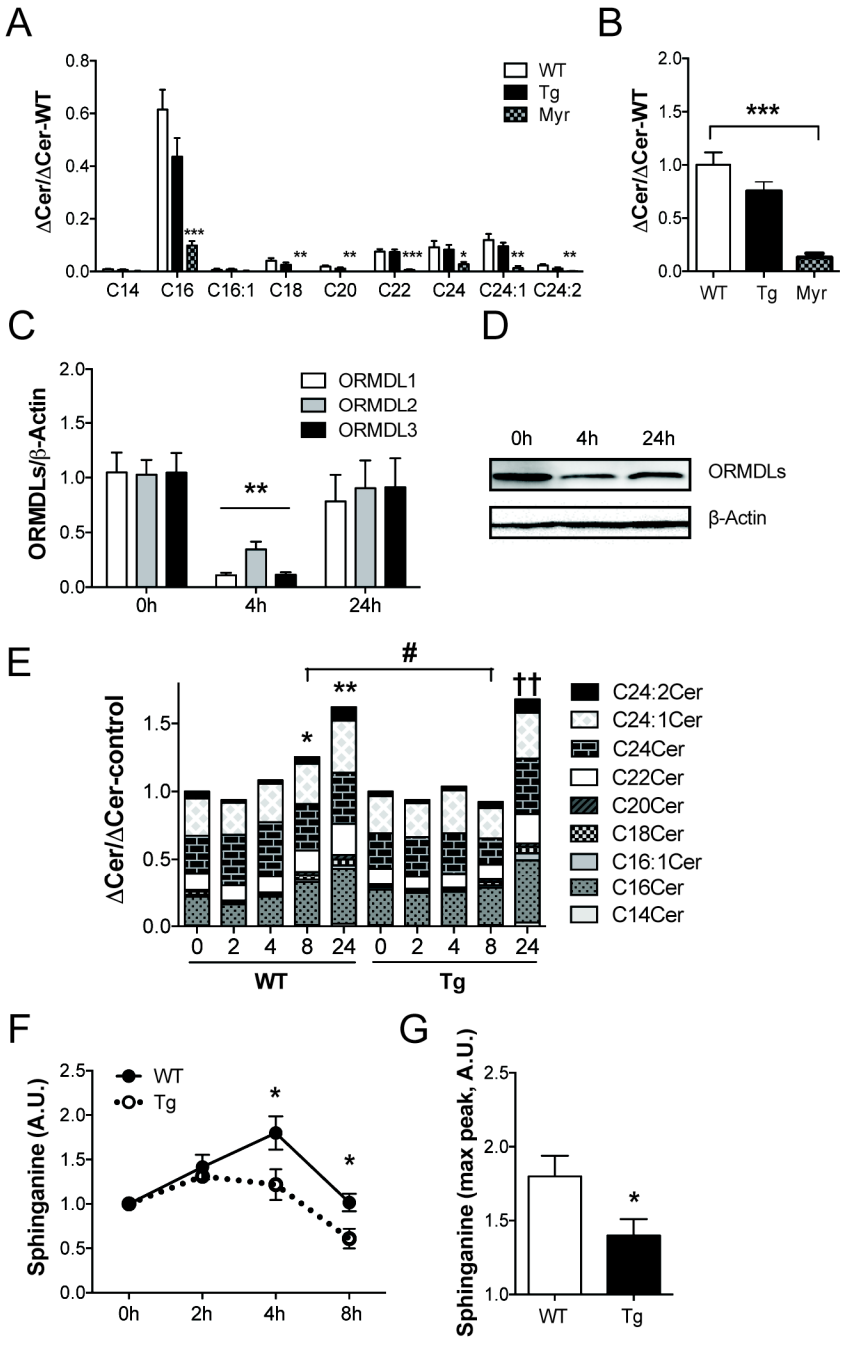


FIGURE 3. Polarization of macrophages in hORMDL3^{Rosa26} mice. Gene expression analyzed by RT-PCR after activation of BMDM from WT and trangenic (Tg) mice at indicated time points. *A-B*, effect of 100 ng/mL LPS and 30 ng/mL INF-γ on M1 polarization of *A*, iNOS and *B* IL-1β. *C-D*, effect of 20 ng/mL IL-4 on M2 polarization. *E-F*, effect of 10 ng/mL IL-4 and 10 ng/mL IL-13 on M2b polarization of *C* and *E*, Arg1, *D* and *F*, CD206. Data are normalized to GAPDH (n = 6-8; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* represent S.E.

FIGURE 4


A
ΔCer/ΔCer-WT
WT
Tg
Myr
C14
C16
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Myr
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ORMDLs/β-Actin
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ORMDLs
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ΔCer/ΔCer-control
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C24:1Cer
C24Cer
C22Cer
C20Cer
C18Cer
C16:1Cer
C16Cer
C14Cer
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F
Sphinganine (A.U.)
WT
Tg
0h
2h
4h
8h
G
Sphinganine (max peak, A.U.)
WT
Tg

FIGURE 4. ORMDL3 modulates basal ceramide content and *de novo* sphingolipid synthesis in activated macrophages. *A-B* and *E-G*, ceramide content in BMDM from WT and hORMDL3^{Rosa26} (*Tg*) mice was quantified by mass spectrometry. *A-B*, Ceramide increase after incubation with 500 µM palmitate (PA) conjugated with 0.5% BSA. A, contribution of the different ceramide species and B, total ceramide. Raw data corrected by sphingomyelin content was normalized to the total ceramide increase obtained in WT macrophages. (*Myr*) WT cells treated with 10µM myriocin 20min before PA addition (n = 8; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *C* and *D*, regulation of ORMDLs after activation with 100 ng/mL LPS of BMDM from WT mice at indicated time points. *C*, gene expression analyzed by RT-PCR of ORMDL1, ORMDL2 and ORMDL3. *D*, representative Western blot of the ORMDL expressional repression. Data are normalized to β-Actin (n = 8; **, $p < 0.01$). *E-G*, BMDM were treated with 100 ng/mL LPS for up to 24h. *E*, time course of total ceramide content in activated BMDM. *Bars* are divided into the different ceramide species detected. Data is normalized to untreated macrophages (*LPS 0h*) (n = 4-8; analysis of WT compared to WT untreated *, $p < 0.05$; **, $p < 0.01$; analysis of Tg compared to Tg untreated ††, $p < 0.01$; analysis of Tg compared to WT #, $p < 0.05$). *F*, time course of sphinganine content in activated macrophages with *G*, the maximal peak of sphinganine. Data is normalized to sphinganine in untreated macrophages (n = 4-8; *, $p < 0.05$). *Error bars* represent S.E.

FIGURE 5

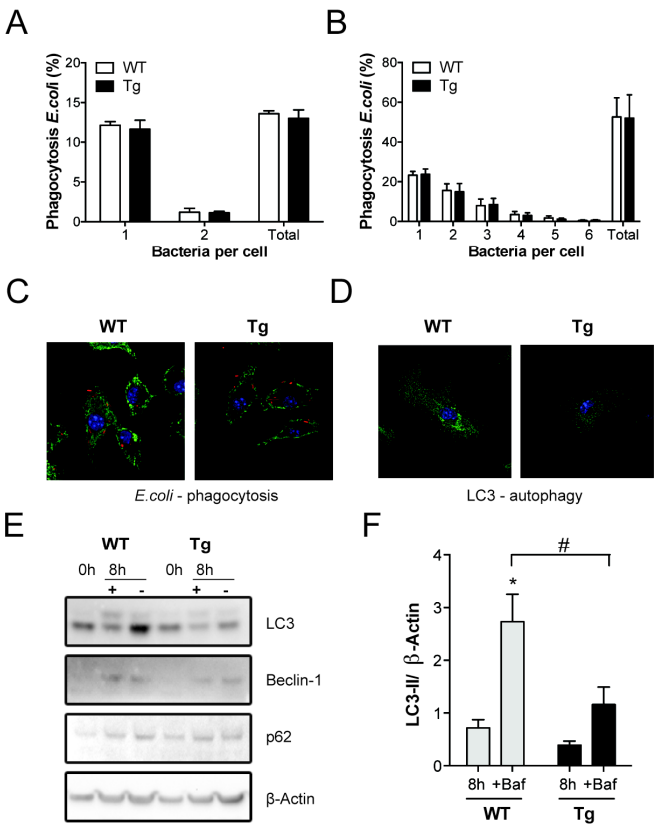


FIGURE 5. Impact of ORMDL3 in early phagocytosis and autophagy in macrophages. *A-C*, intracellular phagocytic uptake of *E.coli* after 30 min incubation in a humidified atmosphere at 37ºC with 5% CO_2. *A* and *B*, quantification of intracellular *E.Coli* per cell at a ratio of *A*, 5:1 and *B*, 20:1 displayed in % in respect to the total number of cells (n = 3). *C*, immunofluorescence staining of fluorescently labeled *E.coli* (*red*) at 5:1 ratio, Phalloidin staining (*green*) marks the plasma membrane and Topro counterstain (*blue*) indicates the location of nuclei. Pictures were taken at a SP8 confocal microscope with a 63x objective. *D-F*, autophagic markers after 100 ng/mL LPS treatment in BMDM from WT and hORMDL3^{Rosa26} (*Tg*) mice. *E*, representative Western blot of LC3, Beclin-1 and p62 after 8h of LPS treatment without and with Bafilomycin A (*Baf*) and *F*, quantification of their expression after 8h of LPS treatment. Data are corrected by β-Actin and normalized to value at time 0h of each group.(n = 4; analysis of WT compared to WT untreated *, $p < 0.05$; analysis of Tg compared to WT #, $p < 0.05$). *Error bars* represent S.E.

FIGURE 6

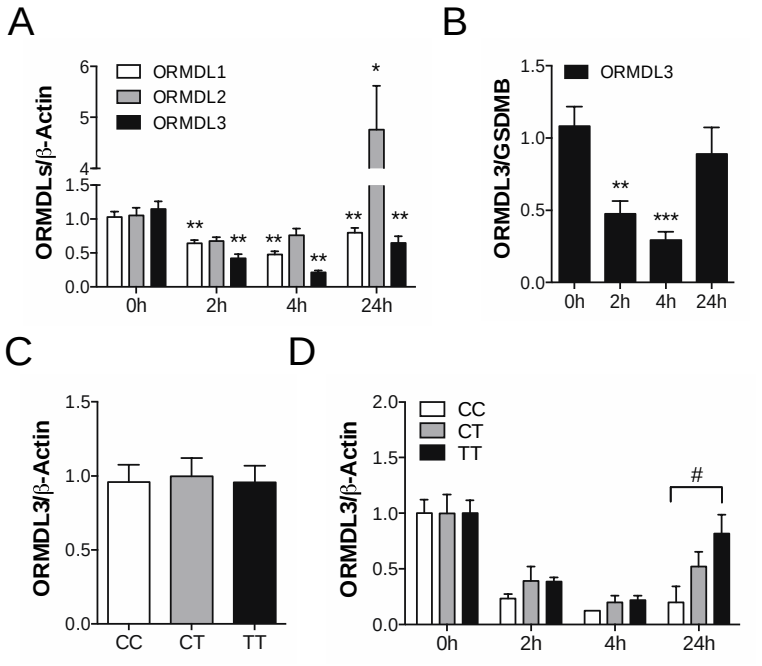
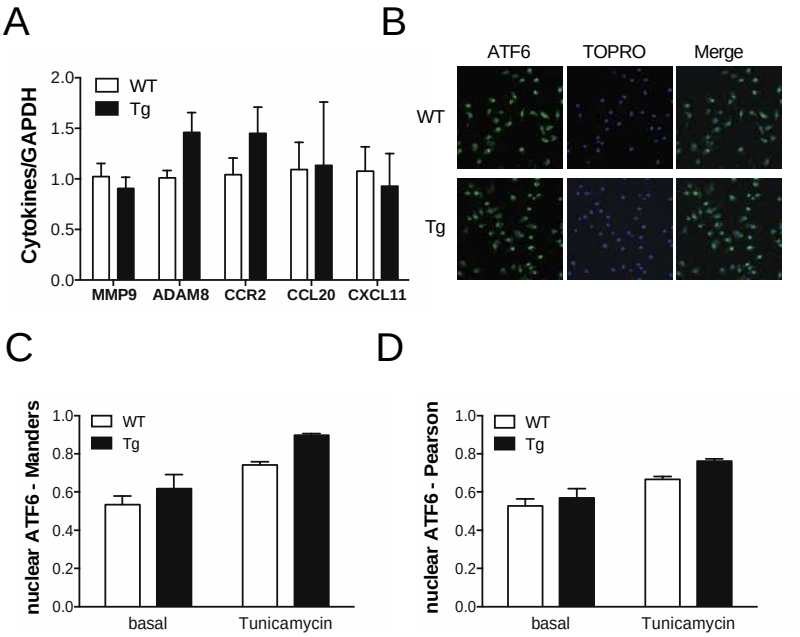


FIGURE 6. ORMDLs are regulated during human monocyte activation. Human blood monocytes from healthy donors were treated with 100 ng/ml LPS for indicated time points. *A-D*, gene expression analyzed by RT-PCR during activation of *A*, ORMDLs normalized to β-Actin or *B*, *ORMDL3* normalized to *GSDMB*. (n = 8-13; **, $p < 0.01$; ***, $p < 0.001$). *C* and *D*, gene expression of ORMDL3 separated by genotype at the disease-associated SNP rs7216389 with the risk allele (*TT*) normalized to β-Actin at *A*, basal level or B, under activation (n = 1-8; analysis of TT carriers compared to CC carriers; #, $p < 0.05$). *Error bars* represent S.E.

FIGURE S1



SUPPLEMENTARY FIGURE S1. ORMDL3 does not alter asthma-associated cytokine expression nor induce ER stress in macrophages. *A*, gene expression analyzed by RT-PCR in BMDM from WT and hORMDL3^{Rosa26} mice of metalloproteases (MMP9, ADAM8), chemokine receptor (CCR2) and chemokines (CXCL10, CXCL11) normalized by GADPH (n = 4). *B-D*, nuclear translocation of ATF6 as a pathway of UPR in BMDM from WT and hORMDL3^{Rosa26} mice. *B*, immunofluorescence staining of ATF6 (*green*) and Topro counterstain (*blue*) indicates the location of nuclei in untreated macrophages. Pictures were taken at a fluorescence microscope with a 63x objective. *C* and *D*, co-localization between ATF6 and the nuclei in untreated or 1 µg/µL tunicamycin-activated macrophages using the JaCoP plug-in of the ImageJ software as represented by the *C*, Pearson coefficient or the *D*, Manders M1/M2 coefficient (n = 5). *Error bars* represent S.E.

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IV. DISCUSSION

After the first successful implementation of GWAS in 2005 [274], the number of studies has increased exponentially and has provided a powerful tool to elucidate genetic causes for complex diseases. By comparison of human genomes in case-control studies, variations in individual nucleotides (SNPs) with a high allelic frequency might be identified as causative for the disease investigated. Asthma is one of those complex diseases, which might be caused by the contribution of environmental factors as well as a genetic background. The starting point of this present thesis was the strong association of the SNP rs7216389 to the risk of developing asthma disease, which was attended by increased *ORMDL3* expression with unknown functions by the time [10]. Over the past years this genetic association has not only been replicated in several GWAS in multiple ethnically diverse populations, showing its robust conformity with an combined odds ratio of 1.37, but was also linked to other inflammatory diseases, such as inflammatory bowel disease, rheumatic arthritis and diabetes type I [2,27–29,36,37]. This led to the hypothesis that ORMDL3 might play a role in the regulation of the immune system, somehow facilitating the development of inflammatory diseases. To date several studies shed light on the functions of ORMDL3 in distinct subsets of the immune system, such as eosinophiles, mast cells and T-lymphocytes, but so far macrophages, a key player in inflammation, have only been characterized rudimentary. To elucidate the cellular function of ORMDLs has been the main focus of other publications including the ones presented in this thesis. The ORMDL family members have been involved in calcium homeostasis, ER stress regulation and *de novo* sphingolipid biosynthesis control. This last one is an important pathway during macrophage activation [273,275], making these cells an extremely promising target for further investigation. Macrophages are cells of the innate immune system that play a central role in early pathogen recognition and cross-talk with other cellular subsets during inflammation.

This thesis compiles two works contributing to the ORMDL3 research field in three different aspects that will be discussed:

- structural insight into the SPT complex and its regulation by ORMDLs
- physiological role of ORMDLs in macrophage physiology
- implication of macrophages in ORMDL3 pathophysiology

Structure and regulation of the ORMDL-SPT complex

The implication of ORMDLs in the *de novo* sphingolipid synthesis via an inhibitory effect on SPT, the first rate-limiting enzyme of this pathway, was initially described in yeast orthologs, namely Orm1 and Orm2 [78,77]. Further studies of the regulatory mechanism revealed a direct interaction between non-phosphorylated Orm proteins and the SPT subunits depending on the cellular sphingolipid content and the activity of the kinases YPK/Npr1 and phosphatases Tap42/Cdc45-PP2A [151,276]. Taken together, Orms act as negative regulators of SPT, thereby establishing sphingolipid homeostasis via a phosphorylation-mediated feedback loop in yeast.

The blockage of SPT by ORMDLs and the resulting reduction in *de novo* sphingolipid synthesis is conserved in the mammalian system, as confirmed by growth arrest reversion after expressing human ORMDL3 in yeast double-KO mutants [1], in which the inhibitory function on SPT was supposed to be mediated via a negative feedback loop [77]. Therefore we wanted to study if the regulation of the mammalian ORMDL-SPT complex formation followed a similar mechanism as described in yeast.

We were able to confirm existing data showing a direct interaction between ORMDL3 and SPTLC1 [154]. Moreover, we demonstrated that ORMDL3 also interacts with SPTLC2, the catalytic subunit of the SPT complex [277]. We clearly proved the existence of a functional ORMDL-SPT complex with a specific coimmunoprecipitation of around 4% for both subunits. However, our results point towards another mechanism than the one described in yeast of a dynamic regulation of the ORMDL-SPT complex modulated by intracellular ceramide levels, as no changes in the extent of ORMDL-ORMDL or ORMDL-SPTLC affinity were observed in high or low sphingolipid conditions. This suggests that the

mechanism of SPT inhibition is not conserved from yeast to mammals. In this respect, the regulation described in yeast was shown to depend on the phosphorylation of a specific N-terminal region in Orms [151,77], which is only present in yeast and plants, whereas vertebrate proteins lack this domain and cluster differently [10]. We therefore proposed a model of inhibition that is not based on the binding affinity between SPT and ORMDLs, but rather on internal rearrangement of the components of this complex. Our FRET experiments presented in the first scientific contribution supported this idea.

Further studies on the stoichiometry of the complex revealed that ORMDLs can form homo- and hetero-oligomers with similar affinities, in which hetero-oligomerization might display slight conformational alterations. Interestingly, the interaction between ORMDL1-ORMDL3 was stronger compared to ORMDL1-ORMDL2 or ORMDL2-ORMDL3, suggesting that the C-terminus of ORMDL2 is placed in further distance from the other isoforms. We also showed a structural rearrangement of the ORMDL homo-oligomers depending on the intracellular sphingolipid content, in such a way that high cellular ceramide levels reduce their interaction between them at the C-terminus. A possible scenario would be that this conformational change allows a more effective inhibition of the SPT enzyme and it would be interesting to evaluate the exact part of the C-terminal domain that undergoes this structural rearrangement and whether it exhibits a sensor for the lipid environment. Although we provided new insight into the regulatory mechanism by which ORMDLs inhibit SPT activity, it remains complex and further functional studies are needed to clarify their interaction.

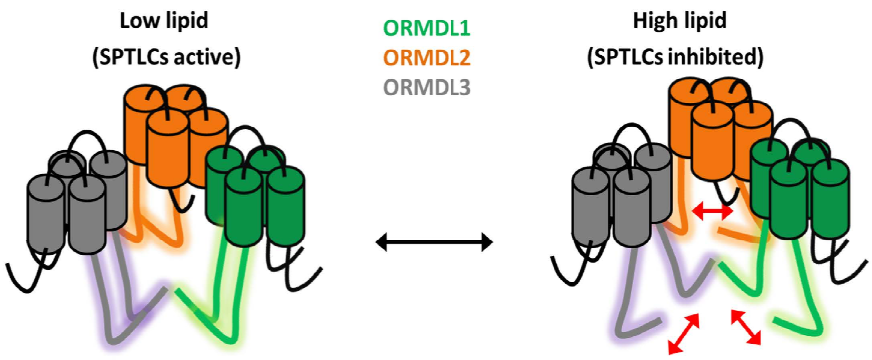

Figure 18: Model for structural rearrangement of ORMDLs. ORMDL homodimers change their conformation depending on the sphingolipid content. Figure extracted from [8]

Within the complex formation the ORMDL proteins were thought to have a redundant function in blocking SPT, since pairwise or single KO was not sufficient to further induce *de novo* sphingolipid synthesis [154]. In this thesis we confirmed that all ORMDL isoforms must be depleted in order to release SPT activity in HEK293 cells. We established and verified a different approach to measure SPT activity not based on radiolabeling, but instead determining the sphingolipid content of cells by mass-spectrometric analysis after palmitate incubation, the enzymatic substrate of SPT. The increase of ceramide levels observed was completely abolished in the presence of myriocin, a specific SPT inhibitor; demonstrating that our protocol allowed us to efficiently monitor sphingolipids from the *de novo* synthesis pathway.

Importantly, we also evaluated the impact of overexpressing individual ORMDL proteins on SPT activity, since the expressional increase of solely *ORMDL3* was associated to the pathophysiology and we wanted to explore a possible link between the two processes. Our results showed that neither increased expression of each individual ORMDL protein nor the

combined overexpression of all three isoforms led to alterations in the ceramide content in this cell line using a heterologous transfection system. In contrast, we did observe major changes in the basal sphingolipid composition in BMDM from our transgenic mouse model with ORMDL3 overexpression (see section 2 for detailed discussion), pointing towards an implication of ORMDL3 expression on the cellular sphingolipid content depending on the expressional system and cell type of study.

Interestingly, triple overexpression of ORMDL proteins completely blocks SPT activity after stimulating the pathway with palmitate. This result reinforced our hypothesis of a structural rearrangement depending on the ceramide content and might display a protective mechanism once ceramides increase to a putative deleterious level. As observed in BMDM, increased ORMDL3 expression only partially reduced the ceramide content after palmitate induction. Therefore we concluded that all three members are needed for an effective sensing and blockage of the *de novo* pathway. However, our results about the inhibition of SPT activity by ORMDLs have to be cautiously interpreted, as they might be partially dependent on the experimental settings, such as cell type, transfection system and palmitate treatment. It is important to state, that in our experiments we have exclusively altered ORMDL expression levels to closely resemble the disease-association, since other components of the ORMDL-SPT complex have not been linked to the pathophysiology.

Physiological role of ORMDLs in macrophages

Macrophages are an important cellular component of the innate immune system and play a central role in early pathogen recognition during inflammation. To date, the role of macrophages in the *ORMDL3*-associated pathophysiology has not been fully elucidated, although these cells are implicated in the development and progression of asthma disease by an inappropriate activation state [278]. According to previous studies, *ORMDL3* expression was upregulated upon bacterial encounter in the RAW264.7 macrophage cell line, as well as in BAL macrophages and eosinophiles [4]. This led us to hypothesize that *ORMDL3* regulation might alter macrophage activation, thereby leading to chronic inflammation and disease state. During the development of this thesis project, a transgenic mouse model was published with an overexpression of *ORMDL3* that showed an increased overall number of macrophages from 8 weeks of age on [6].

Macrophage activation is a physiological process of particular relevance regarding the role of ORMDL3, since microbial LPS stimulation leads to increased intracellular ceramide levels, which mainly originate from *de novo* sphingolipid synthesis at early time points [273,279–281]. Interfering with this increase of *de novo* ceramides generated via the SPT pathway leads to defective macrophage activation and impairs other important processes, like autophagy and pro-inflammatory cytokine production [273,275].

Since macrophage activation is an extremely sensitive process, we studied the impact of ORMDL3 on macrophage physiology in BMDM from transgenic mice, which constitutively overexpress the human *ORMDL3* gene under the endogenous *Rosa26* locus, thereby avoiding possible artifacts caused by transient transfection. Our model showed around three fold induction of ORMDL3 protein expression in macrophages compared

to WT cells, which were considered as significantly higher levels. In a previous study using Fenretinide that was shown to block *de novo* sphingolipid synthesis after pathogenic RAW264.7 activation, the same TNF-α levels, but decreased IL1-β and IL-6 expression were observed [275]. Since ORMDL3 was thought to similarly affect *de novo* sphingolipid synthesis, we hypothesized that higher levels might alter the cytokine expression profile. However, comparing selected pro-inflammatory activation markers after LPS exposure showed unaltered induction and temporal regulation in our model. These parameters were also subject to a study in RAW264.7 macrophages obtaining no differences in cytokine expression after ORMDL3 transfection [156]. But they claimed that cytokine expression is significantly induced by LPS treatment when RAW264.7 cells contain an aberrant amount of ORMDL3 expression of up to five fold [156]. The question for the exact pathophysiological levels of ORMDL3 remains difficult to determine, since the influence of the risk allele was shown to be cell type specific [10,21,25,42]. Besides, there is no data about protein levels depending on the SNP. Finally, no changes in the asthma-related cytokine expression levels of IL-6 and IL-8 were observed in airway epithelial cells after LPS stimulation by ORMDL3 knock-down [80]. All these evidences led to the conclusion, that moderately increased ORMDL3 levels do not alter the activation state of macrophages, as indicated by unaltered pro-inflammatory cytokine expression after LPS activation.

Although we did not observe major differences in macrophage activation, we wanted to explore whether an imbalance of the **macrophage polarization** process might be the missing link between ORMDL3 expression and pathology. Asthma is traditionally referred to as a T-helper-2 (Th-2) cell driven inflammatory disorder [282,283], but this basic characterization is about to change due to the immense heterogeneity of

the disease, where imbalance of M1/M2 polarization was attributed to different macrophage subsets for example in bronchial asthma [284]. Interestingly, asthma has also been associated to alterations in temporal and spatial pattern of inflammatory gene expression [285]. However, macrophages with higher levels of ORMDL3 showed equal ability to polarize towards the M1 or M2a phenotype. The basal cytokine expression profile showed no alterations, discarding the possibility that ORMDL3 induces a profound imbalance in macrophage subsets. Interestingly, intranasal administration of the cytokines IL-4 and IL-13 have been shown to increase *ORMDL3* expression levels in bronchial epithelium of WT mice [4]. In our hands, stimulation of BMDM from WT mice with IL-4 or the combination of IL-4 and IL-13 did not change the *ORMDL3* expression levels (data not shown), suggesting that this pathway might be cell type-specific mediated via a downstream mechanism of cytokine administration.

During this thesis project, several studies were published trying to elucidate different aspects of the mechanistic regulation underlying the inhibitory effect of ORMDL3 on SPT. Although ORMDL proteins were first described as intracellular sphingolipid sensors and negative regulators of the rate-limiting enzyme of ***de novo* sphingolipid synthesis** in 2010 [77], this regulation in macrophages had not been addressed *in vivo* yet. Whether increased levels of *ORMDL3* expression produced by a SNP that exert a *cis* regulation can be related to its specific function in sphingolipid synthesis is of particular interest for explaining the pathophysiological association. At that point, no alterations in basal sphingolipid levels have been described, as evaluated by knock-down [154] and in our individual overexpression studies (see section 1 for detailed discussion) [277]. Only recently ORMDL3 expression was attributed to significant alterations of ceramide species in RAW264.7 cells, which resembles our working model

of macrophages more precisely [156]. Therein, high levels of ORMDL3 led to decreased *de novo* sphingolipids levels, mainly with a C16-, C22-, and C24-backbone. In accordance with our results, BMDM from transgenic mice clearly display alterations in the cellular ceramide homeostasis with a reduction in rather long-chain ceramide species. One of the enzymatic reactions in the *de novo* pathway downstream of SPT is carried out by (dihydro)-ceramide synthase (CerS), which acylates sphinganine to dihydroceramide by addition of a fatty acyl-CoA. In mammals six different CerS have been described with a specific acyl-CoA preference, thereby producing a specific dihydroceramide profile [108,109]. Of particular interest herein are CerS1, which mainly generates C18-ceramide species [110] and CerS2/CerS3 by producing middle to long chain acyl-CoA ceramides (Laviad et al. 2008; Mizutani et al. 2006). We therefore speculate, that ORMDL3 expression levels might additionally regulate the activity of at least one of the CerS, suggesting a more complex scenario of *de novo* ceramide inhibition. Regarding more complex sphingolipids, elevated levels of dihydrosphingosine-1-phosphate and sphingosine-1-phosphate were detected after ORMDL3 knock-down [155], which implies the induction of other routes in the ceramide pathway and the metabolizing enzymes, such as ceramidases and sphingosine kinases. It also suggests to interpret increased ceramide levels after ORMDL depletion cautiously, as they might be underestimated due to the consumption of the dihydrosphingosine precursor. However, differences in sphingosine-1-phosphate levels could also originate from the ceramide degradation pathway, since the sphingosine backbone is degraded via this species. Once ceramide is formed, it may be subjected to degradation by one of the ceramidases and the resulting sphingosine backbone is also degraded through the action of sphingosine-1-phosphate and sphingosine-1-phosphate lyase. Considering all these evidences, it is likely that ORMDL3 affects several pathways in the ceramide homeostasis, as it

displays a tightly regulated enzymatic network.

Stimulation of the *de novo* ceramide synthesis with palmitate in BMDM from transgenic mice only partially blocked SPT activity, which is in accordance with previous results in HEK293 cells, where the overexpression of all three ORMDL isoforms was necessary to completely inhibit SPT activity [277]. This reinforces our idea of a regulatory mechanism based on structural rearrangement. In conclusion, ORMDL3 is able to lower the homeostatic content of ceramides in macrophages, but not sufficient to effectively block SPT activity. Myriocin, a specific pharmacological inhibitor of SPT, has been used to mimic the pathophysiological role of ORMDL3 in past studies, but our results argue against the idea that myriocin resembles the same effect as ORMDL3 [176]. In this regard, we specify that myriocin treatment is a valid control condition for studying the ORMDL-SPT complex, but not for ORMDL3 individually.

Another important finding in the presented thesis is the **regulation of the ORMDL family expression** modified in a coordinated manner, thereby controlling *de novo* sphingolipid levels upon bacterial activation with LPS in macrophages. The initial results obtained in the RAW264.7 macrophage cell line were reproduced not only in BMDM from WT mice, but also in human monocytes extracted from blood samples of healthy donors, demonstrating the importance of this conserved regulation amongst various cellular settings. As mentioned before Miller and colleagues have shown an upregulation of ORMDL3 in RAW264.7 macrophages, but neither timing nor concentration of LPS stimulation have been stated, leading us to the assumption that incubation times were around 24 h, as we observed higher expression levels of ORMDL3 at this time point [4]. In detail, in RAW264.7 macrophages we were able to

correlate the repression of all three ORMDL family members after early cell activation with the induction of *de novo* ceramide synthesis, proposing a model under which expressional reduction of ORMDLs release the inhibitory function on SPT. Our results strengthen the previous observation that all three ORMDL proteins must be depleted in order to release SPT activity [154], and moreover bring their regulation into a physiological relevant context. Since LPS triggers a signaling pathway via TLR4, it would be interesting to study whether ORMDLs also undergo a coordinated regulation by other stimuli, such as gram-positive bacteria or viral infections signaling via different TLRs on the plasma membrane.

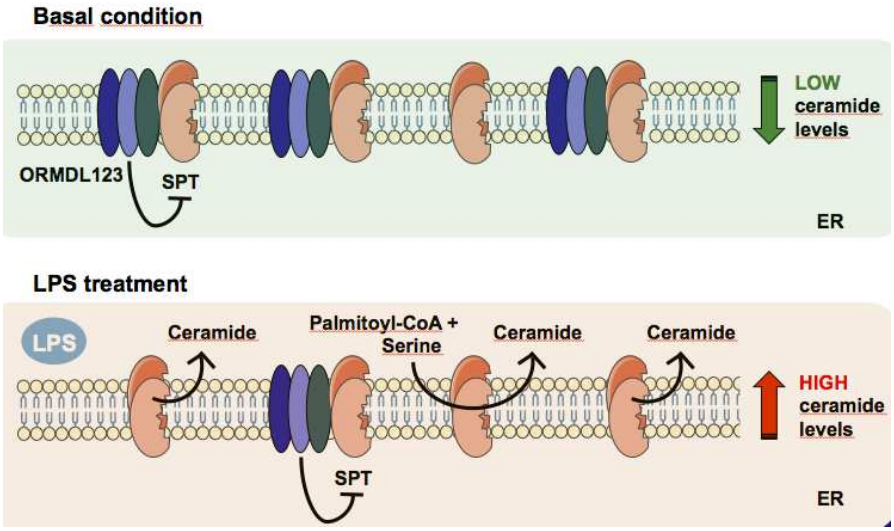

Figure 19: Model of ORMDLs regulation under activation. Coordinated regulation of the ORMDL proteins under activation allows the SPT activity release.

Having in mind that macrophage activation is an extremely interesting pathway to study due to the correlation of increased ceramide levels after LPS stimulation generated via the *de novo* sphingolipid pathway [273,275], we decided to specifically study the impact of ORMDL3 in this process.

We used BMDM from our transgenic mouse model, in which human ORMDL3 expression is under the control of an exogenous promoter and thereby not altered by activation stimuli. Upon LPS stimulation we detected an inhibitory effect of ORMDL3 on the total ceramide content, but more importantly also on *de novo* sphingolipid synthesis as measured by sphinganine. In conclusion, our work shows that impairment in the coordinated downregulation of ORMDLs prevents SPT activity release in response to bacterial encounter. One possible mechanisms affected might be apoptosis, since pro-apoptotic functions have been linked to elevated ceramide [286], but was not subject of this study.

However, we monitored the two membrane-sensitive processes, namely phagocytosis and autophagy to study consequences of altered membrane composition induced by changes in ORMDL3 expression. Since the crucial role of *de novo* sphingolipids in **phagocytosis** of fungal *candida albicans* was demonstrated in macrophages [240], we thought ORMDL3 might play a role therein. In our experimental settings, we did not detect differences in phagocystosed *E. coli* after an incubation time of 30 min of low and high bacteria to cell ratios. Given our experimental settings, we would like to include a myriocin control as in the study by Tafesse and colleagues, and also increase the incubation time between infection and fixation. We hypothesize that although we have the same initial infection rate after bacterial exposure, we might able to see differences in further process of autophagy and degradation. As mentioned before, **autophagy** induction after LPS exposure also depends on *de novo* ceramide synthesis. Increased ORMDL3 levels impaired autophagy induction, specifically at the level of autophagosome elongation. However, further studies are needed to elucidate the exact mechanism by which altered sphingolipid content induced by ORMDL3 can lead to such an autophagic impairment and the consequences for macrophage physiology. Dysregulation of

autophagy after bacterial infection might lead to a negative outcome of bacterial clearance and thereby sustained inflammation [256]. It is striking that defective autophagy was not altering the M1 subset polarization process, since abnormal accumulation of non-functional material in the cell induces a pro-inflammatory program [287]. In this sense, a general study of autophagy induction by other stimuli like starvation should be conducted to have a better understanding of this issue. Regarding the context of pathophysiology, it is interesting to point out that one of the known causes of inflammatory bowel diseases, to which *ORMDL3* has been associated, is an anomalous autophagic process in the gut [288,289].

Macrophages in ORMDL pathophysiology

The association between ORMDL3 and asthma has been independently reproduced in several GWAS and non-GWAS studies and gained attention of the scientific community, because asthma is a complex disease caused by both environmental factors and genetic predisposition, which affects around 300 million people worldwide. GWAS are a powerful tool to elucidate candidate genes or chromosomal regions that can be associated to diseases in a large cohort size. However, this approach is being criticized for false-positive results and for genes that have been initially linked to diseases by GWAS further functional studies are needed to assure their implication. There are many studies exploring the genetic region associated to these SNPs in human populations, but fairly few that evaluate the functional impact of ORMDLs in cell physiology.

Two publications from Miller and colleagues focused on the effect of ORMDL3 expression in asthma [4,6]. In the latter study a **transgenic mouse model** overexpressing ORMDL3 was generated and several characteristics of asthma were reported, such as spontaneous airway remodeling and hyperresponsiveness. This phenotype was explained by the induction of the ATF6 pathway and increased expression of SERCA, which led to enhanced production of metalloproteases and subsequent matrix remodeling. However, there are contradictory results regarding the importance of SERCA, as decreased activity has been previously shown in airway remodeling [290]. In this mouse model the authors claimed that the mechanism of the airway remodeling was not dependent on increased airway inflammation and the early events detected were in the lung epithelium.

Another approach to study the relationship between ORMDL3 and asthma via sphingolipid synthesis has been performed in mice by myriocin inhalation and heterologous SPTLC2 knock-out [176]. In both experimental settings decreased *de novo* sphingolipid synthesis was detected, which was described as causative for bronchial reactivity in the absence of inflammation. Airway hyperreactivity was observed as a consequence of imbalanced magnesium homeostasis in bronchia. As mentioned above, we argue against the usage of myriocin in order to mimic ORMDL3 overexpression since individual isoform overexpression does not resemble the same effect of a SPT inhibitor. Remarkably, the knock-out mouse model for both SPTLC subunits showed embryonic lethality.

Our ORMDL3 transgenic mouse model underlay several different features compared to the one by Miller and colleagues; most importantly we introduced the human *ORMDL3* gene into the endogenous *Rosa26* locus, thereby tightly controlling the locus of gene insertion. We observed a general increase of protein expression in various tissues including lung, spleen and BMDM and validated the correct targeting by localizing the protein to the endoplasmic reticulum. However, in our mouse model we did not observed spontaneous airway remodeling in mice up to 12 weeks of age. In fact, it is very unusual for a mouse to develop a spontaneous asthma phenotype [291]. Probably, the strategy for generating the mouse model is behind these discrepancies. Therefore we decided to focus on other cell types in order to elucidate the impact of ORMDL3 overexpression. In this thesis we have studied the consequences of higher ORMDL3 expression levels in macrophage physiology in an attempt to link the risk allele to asthma with an imbalance in innate immunity.

As presented above we observed reduced ceramide content in basal macrophages and impaired *de novo* synthesis in activated macrophages, since the expression of the human *ORMDL3* is under the control of an

exogenous promoter interfering with the coordinated downregulation. The result of this lipid imbalance is the impairment in the elongation phase of the autophagic process. This outcome could explain the mechanism of ORMDL3 pathophysiology since defective autophagy has been previously linked to inflammatory conditions [256].

We have focused on the **SNP rs7216389**, which is located within the first intron of the neighboring *GSDMB* gene in cis-regulation with *ORMDL3* [21]. Genetic variation at this SNP (T allele) was shown to **increase *ORMDL3* transcript levels**, thereby determining the susceptibility to childhood asthma [10]. We therefore wanted to explore the implication of ORMDL3 in human monocyte activation. Surprisingly, in our samples of human monocytes we did not observe changes in *ORMDL3* expression. This could be caused by the sample size, but it could also reveal that the strength of these genetic factors might vary depending on the cell type. If this is case, the idea of increased ORMDL3 expression levels in macrophages should be taken cautiously.

GWAS studies have mainly linked the genetic region around *ORMDL3* gene to inflammatory diseases. This is surprising, regarding the structural homology of about 80% between the three ORMDL family members. It could be explained by the absence of *cis* regulatory elements controlling the transcript levels in the genomic regions of *ORMDL1* and *ORMDL2*, of which each is located on different chromosomes. Alternative explanations include a tissue-specific expressional regulation or a structural difference of ORMDL3 that is lacking in the other two isoforms. However, as discussed before, regarding their function in *de novo* sphingolipid synthesis all three members are needed for complete inhibition of SPT activity.

The scenario of the genomic region around *ORMDL3* and its implication

is getting more complex with the accumulation of genetic studies. Other SNPs have been described at chromosome 17 (17q12-21), such as SNP rs1293623, which also exerts cis-regulation for *ORMDL3* and the surrounding genes *IKZF3*, *ZPBP2* and *GSDMB*. In fact, lately the term regulatory haplotype has been used to define the combination of SNPs that determine the gene expression in the region [21]. Since there are several genes under control, we should consider a more complex scenario where the pattern of expression regulation of several genes in that region might account for the association studies. Moreover, whereas one of the haplotypes induces expression of ORMDL3/GSDMB and has been associated to asthma disease, the opposite haplotype with decreased ORMDL/GSDMB levels was associated to Crohn disease (CD), type 1 diabetes (T1D) and primary biliary cirrhosis (PBC) [21]. Interestingly, CD has been linked to disturbances of the innate immune response mainly via ER stress and UPR induction, epithelial barrier dysfunction and autophagy, which could all possible interconnect it to ORMDL3 [292]. However, the haplotype associated to CD would be determined by a lower ORMDL3 expression.

There was another important question derived from our studies with the transgenic mouse model. We observed an alteration in autophagy, when *ORMDL3* was not under control of the coordinated downregulation during macrophage activation. An extrapolation to the human scenario would be a differential *ORMDL3* regulation during activation depending on the regulatory haplotype around *ORMDL3*. Our results showed that the downregulation of the ORMDL3 expression happens equally for C or T carriers at the SNP rs7216389. On one hand, this argues against the pathophysiological relevance of the impairment in autophagy observed in transgenic macrophages. On the other hand, it reinforces the idea that this regulation is also important in human monocytes. The differences between

alleles observed at 24 h cannot be related to SPT release, but to the cellular sensing mechanism of ceramide content. However, the experimental design and thereof extracted data limits further conclusions in this respect.

In summary, this thesis explores the functional impact of ORMDL3 in macrophage physiology, based on the notion that increased expression levels have been associated to a higher risk to develop several inflammatory/autoimmune diseases. We elucidated the importance of ORMDLs as negative modulators of SPT activity, especially in macrophage physiology. Our transgenic mouse model showed reduced ceramide content and altered *de novo* synthesis under macrophage activation, resulting in impaired autophagy, an alteration that could contribute to further understanding in the ORMDL3 pathophysiology. However, our data in human monocytes challenges the contribution of macrophages in *ORMDL3*-associated pathophysiology, since the coordinated *ORMDL* downregulation during activation is not dependent on the genetic components around the *ORMDL3* locus. Our work adds further understanding of the role of ORMDLs in macrophage physiology and provides insights on ORMDL3 pathophysiology.

V. CONCLUSIONS

1 ORMDL interaction with both subunits of the SPT complex (SPTLC1 and SPTCL2) is independent of cellular ceramide content.

2 Cellular ceramide composition produces structural rearrangement between ORMDL homodimers at the C-terminal tails.

3 Triple but not individual ORMDL subunit overexpression blocks SPT activity.

4 Basal cellular sphingolipid content is modified by increased ORMDL3 expression dependent on cell-type and expressional system.

5 The expression of all three ORMDL isoforms is coordinately downregulated upon LPS activation in macrophages. Interference of this regulation by exogenous promoters prevents SPT activity release.

6 Increased ORMDL3 levels do not alter the activation state of macrophages and their ability to polarize towards M1/M2 phenotypes.

7 Increased ORMDL3 levels impair autophagy at the level of autophagosome elongation.

8 Genetic variation at the asthma-associated SNP rs7216389 does not lead to alteration in ORMDL3 expression in human monocytes, neither to changes in the expressional regulation upon activation.

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